YEAST PHYSIOLOGY AND FLAVOUR FORMATION DURING PRODUCTION OF ALCOHOL-FREE BEER.

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YEAST PHYSIOLOGY AND FLAVOUR FORMATION DURING PRODUCTION OF ALCOHOL-FREE BEER.

Proefschrift

ter verkrijging van de graad van doctor op gezag van de rector magnificus van Wageningen Universiteit, Dr C.M. Karssen, in het openbaar te verdedigen op woensdag 1 december 1999 des namiddags 13:30 uur in de Aula.

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Cover: 'The Brewmaster's Respite' by Eduard Grützner (1846 - 1925).

STELLINGEN

1. In *S. cerevisiae* spelen, onder anaërobe omstandigheden, NADP-afhankelijke alcohol dehydrogenasen een belangrijke rol in de redox-balans van NADPH/$NADP^+$.
   Dit proefschrift.

2. Het immobilizeren van gist op een drager verbetert zijn 'overall performance' tijdens de productie van alcoholvrij bier.
   Dit proefschrift.

3. De bewering dat gisten niet delen in een suikervrij medium is onjuist.
   Van Hamersveld *et al.* 1996. J. Inst. Brewing **102**: 333-342.

4. Op grond van de door Younis en Stewart gebruikte proefopzet is de interpretatie van de activiteit van ester-synthetizerende enzymen en estervorming niet gerechtvaardigd.
   Younis and Stewart. 1998. J. Inst. Brewing **104**: 255-264.

5. Gedrag, waarmee het verspillen van energie gestimuleerd wordt, biedt een zeker evolutionair voordeel en is dus onderhevig aan natuurlijke selectie.
   Russell and Cook. 1995. Microbiol. Rev. **59**: 48-62.

6. De smaakstabiliteit van pilsner bier wordt in hoge mate bepaald in de mouterij.

7. Het menselijk vermogen om getalgrootte in te schatten is logarythmisch van aanleg.

8. Bij het aanleggen van nieuwe wegen om het file-probleem op te lossen wordt stelselmatig vergeten dat er een wet bestaat tot het behoud van rijtijd: 'De afstand in woon-werkverkeer neemt toe, naarmate de rijtijd afneemt'.

9. Kroegbezoek is als de hartslag; de frequentie kan variëren maar regelmaat is noodzaak.

10. Het oudste beroep van de wereld is genoegzaam bekend; het oudste bedrijf van de wereld is ongetwijfeld het familiebedrijf.

Stellingen behorend bij het proefschrift 'Yeast Physiology and Flavour Formation during Production of Alcohol-free Beer'.

Martijn van Iersel, 1 december 1999.

aan ons pap
aan ons mam

VOORWOORD

Laat ik beginnen met een promotie-onderzoek te vergelijken met een wandeltocht. Er zijn in de loop van de tijd een aantal moeilijk begaanbare paden, je kunt de weg kwijt raken, meerdere wegen leiden naar hetzelfde doel, en succes bereik je niet alleen. Aan het eind van de tocht wil ik dan ook alle mede-wandelaars bedanken.

Frans Rombouts en Tjakko Abee, bedankt voor jullie rol als gids. Frans was voortdurend gericht op het einddoel, terwijl Tjakko als theoreticus de nieuwe paden ontdekte. Kortom, een goede combinatie!

De afgelegde route werd vanuit Bavaria nauwgezet gevolgd. Wim Swinkels en Ben van Dieren wil ik bedanken voor de zeer praktijk-gerichte opmerkingen en aanvullingen en Eric Meersman voor de ondersteuning en discussies vanuit het laboratorium.

Eén van de bewandelde paden voerde naar de vakgroep Biochemie, waar, in samenwerking met Willem van Berkel en Michel Eppink, een nieuw enzym werd gezuiverd. Deze aftakking van het hoofdpad leidde dan ook tot een mooie, gezamelijke publicatie!

Tijdens de tocht heb ik een aantal malen kort gezelschap gehad. De studenten Irmgard Pijpers, Sandra Hermsen, Peter Ybema, Jasper van Deurzen, Marjoleine Arntz, Ellen Brouwer-Post en Stijn Swinkels bewandelden verschillende paden en verkenden de route. Zonder hun werk had u dit niet gelezen.

Voor wandeling en werk heb je de juiste 'tools' nodig. Het was dan ook telkens weer een plezier af te dalen naar de 1^{e} verdieping, waar Jan Cozijnsen met grote kundigheid de GC's draaiende houdt. Jan bedankt!

Collega-wandelaars zorgden ervoor dat het goed toeven was op de 4^{e} verdieping. I'ld like to thank Aidan Coffey for learning me the value of working late, in order to be able to take a pint or two afterwards....

Publiek had ik natuurlijk ook; er zijn gelukkig altijd vrienden en naaste familie die 'tot vervelens toe' blijven informeren naar de voortgang. Zij maakten dat na een donker bos en diep dal het uitzicht extra mooi was.

Eenmaal uit het bos gekomen had ik een mooi uitzicht op het eindpunt; dat echter verder weg bleek te liggen dan gedacht. Sabine bedankt voor de steun, en voor het geduld wanneer ik telkens opnieuw weer achter de computer kroop. Op het eind van de tocht kwam er, van een klein opgewekt kaboutertje, plotseling nog een duwtje in de rug. Lieke, ik hoop dat ik net zoveel steun voor je kan betekenen tijdens jouw wandeling als jij voor mij bent geweest!

ABSTRACT

Production of alcohol-free beer is performed with immobilized cells of *Saccharomyces cerevisiae* var. *uvarum*. In the reactor, combined stress factors such as low temperature (0-4°C) and anaerobic conditions limit cell metabolism.

Cells of *S. cerevisiae* are able to grow as low as -2°C. Although sugar metabolism is instantaneously and similar in cells grown at high or low temperature, respiration differs significantly. Latter cells do not show substrate-induced respiration in spite of the presence of active mitochondria and biomass yield was substantially reduced.

We noticed an increase in reductive capacity of yeasts grown under anaerobic conditions and we subsequently purified and characterized a novel NADP-dependent branched-chain ADH. Based on the high reductive activity with 3-methylbutanal at physiological pH and higher ionic strength, the enzyme will have an important function in reduction of Strecker aldehydes during alcoholic fermentation.

The activity of the bcADH and the influence of immobilization on cell physiology were further monitored during alcohol-free beer production. Higher activities of glycolytic enzymes, and the bcADH were observed compared to those in batch grown cells. In addition, glucose flux was increased. The shifts in enzyme activities and glucose flux correlate with a higher *in vivo* reduction capacity of the immobilized cells.

Formation of ester and diacetyl by immobilized yeast cells were investigated. Due to the anaerobic conditions, acetate ester production and *in vitro* enzyme activity increase simultaneous with the decrease in unsaturated fatty acids. Sterol metabolism is blocked; consequently, squalene accumulates. Despite the presence of active acetohydroxy acid synthase, no diacetyl-precursor is formed at low temperatures.

S. cerevisiae W34 flocculates at the end of the exponential growth phase. Grown at low temperatures, cells have a higher flocculation capacity and a higher cell wall hydrophobicity. The immobilization of cells to DEAE-cellulose is influenced by both charge differences between cell wall and carrier and flocculation characteristics.

In conclusion, immobilized cells show some unique physiological features at low temperatures. These contribute positively to the limited fermentation of wort to alcohol-free beer.

LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase
bcADH	branched-chain alcohol dehydrogenase
BisTris	bis[2-hydroxyethyl]-imino-tris[hydroxymethyl]-methane
CE	cell extract
cFDA	carboxyfluorescein diacetate
cfu	colony-forming unit
CHES	(2-[N-cyclohexylamino]-ethanesulfonic acid
CSTR	continuous stirred tank reactor
DASPMI	2-(4-(dimethylamino)-styryl)-N-methylpyridinium iodide
DEAE	di-ethyl-amino-ethyl
DMSO	dimethylsulfoxide
DTT	dithiothreitol
DW	dry weight
FBR	fluidized bed reactor
GC-HS	gas chromatography of the static headspace
HPAEC	high-performance anion-exchange chromatography
l.a.b.	lactic acid bacteria
MES	2-[N-morpholino]-ethanesulfonic acid
MOPS	3-[N-morpholino]-propanesulfonic acid
TCA	tricarboxylic acid cycle
TRIS	tris[hydroxymethyl]-aminomethane
TPP	thiamin pyrophosphate
OD	optical density
PBR	packed bed reactor
PPP	pentose phosphate pathway
VDK	vicinal diketones

CONTENTS

GENERAL INTRODUCTION

Part of this chapter was published by: M.F.M. van Iersel, E. Meersman, W. Swinkels, T. Abee, and F.M. Rombouts. 1995. Continuous production of non-alcohol beer by immobilized yeast at low temperature. J. Indust. Microbiol. 14: 495-501.

Beer brewing: history

The art of brewing beer has developed over a period of 4000 to 6000 years. Remains of ancient Babylonian and Egyptian civilizations bear inscriptions and pictures referring to the brewing of beer. A great variety of techniques and methods have been developed, probably independent from each other. Over the millennia, different types of beer have been developed but several features are common in all. The presence of alcohol and the low pH make it a relatively safe product from the microbiological point of view. In addition, beer has a good nutritional value, a high caloric content, a variety of amino acids, vitamins of the B complex and has a pleasant flavour. Due to these characteristics, beer has been the most popular drink for centuries. In the Middle Ages an annual consumption of approx. 400 litres per capita has been estimated. Beer consumption dropped, but raised again after the development of lager beer at the end of the 19th century. Nowadays, beer is still a very popular drink in Western Europe, although consumption is at a stable level or slightly declining.

Production of lager beer originated in the area of the Czech Republic and Southern Germany. Its increase in popularity in other European countries was only possible with the simultaneous development of cooling systems (ammonia evaporation). Fermentation under these conditions produced a superior beer in the sense that it was clear (no yeast deposit), had a pleasant hop aroma and bitterness, a beautiful golden transparent colour, and constant quality due to less severe microbiological infections during fermentation in the temperature-range of 8 to 12°C. Pilsner beer thus became the standard beer in the major part of Europe during the 20th century. Only recently, the number of new beers has increased substantially. Some are based on the older and traditional method of top-fermentation and addition of herbs instead of hops. Others, such as the alcohol-free or low-alcohol beers, are based on new technological developments.

Production of beer

Beers are brewed with malted barley, often mixed with other cereals either malted or unmalted. Malted barley can be considered a package of enzymes and starch, which have to be liberated by milling and mashing. Glucanases degrade cell wall glucans, amylases liberate sugars from starch and several proteases are necessary to degrade proteins into peptides and amino acids. Since these enzymes have different activity curves with respect to pH and temperature, it is necessary that both parameters are controlled well during mashing. The mash is subsequently filtered over a lauter tun or through a thin bed filter and a clear extract is gained, which is called wort. The wort is boiled during which hops are added to give the beer its bitterness and hop flavour and subsequently, it is cooled

down to 8 to 12°C and yeast is added. During fermentation, sugars in the wort are converted to ethanol, and carbon dioxide. In addition, a great variety of flavour components is formed by yeast metabolism. After a maturation period, the beer is filtered and finally bottled.

Yeast

Generally, yeasts play an important role in the fermentation of beverages. Examples are fermentation of milk to kefir (Eastern Europe), palm tree sap to Tuwak (Indonesia), and grapejuice to wine (Table 1).

Table 1 Fermented beverages.

Product	Origin	Substrate	Organisms
Beer		malted barley	
Lambic	Belgium	and (un)malted	*Enterobacteriaceae*, yeasts, *Pediococcus*
Weissbier	Germany	cereals (a.o.	*S. cerevisiae*, lactic acid bacteria
Ale	W. Europe	rice, wheat,	*S. cerevisiae* (top-fermenting)
Lager	Czech Rep., Bavaria	maize)	*S. cerevisiae* (bottom-fermenting)
Kefir	E. Europe	milk	yeasts, lactic acid bacteria
Sake	Japan	rice	*Aspergillus*, *Saccharomyces*, l.a.b.
Pulgue	Mexico	agave	*Zymomonas*, yeasts, l.a.b.
Kaffir	Africa	sorghum, maize	yeasts, l.a.b.
Wine	S. Europe	grapes	yeasts, l.a.b.

l.a.b. lactic acid bacteria

Yeast in these fermentations originate primarily from the production environment, although with wine, more and more producers add yeast cultures in order to have a better control of the process. This is also the case with beer, which is generally fermented with a well characterized yeast strain. An exception here are the Lambic beers, which are brewed in close vicinity of Brussels, Belgium, and are fermented without addition of a specific yeast culture. The very long fermentation periods of sometimes over one year, and the non-sterile conditions give that a variety of species can be found. Strong fermenting *Saccharomyces* species are responsible for the first alcoholic fermentation. Later, a combination of lactic acid bacteria, *Enterobacteriaceae* and yeast species such as *Brettanomyces bruxellensis* and *Br. lambicus* ferment the oligo and polysaccharides. The result is a beverage with a high concentration of acids, esters and alcohol, which is blended with sugars and young Lambic to improve its taste.

The importance of yeast in fermentation was first recognized by Pasteur in the 19th century and written down in his famous 'Etudes sur la Bière' (1876). Hanssen in 1888 was the first to isolate specific yeast strains, and

propagate them in pure culture. Variants there-off are nowadays used for the majority of lager beer production in the world.

Traditionally, brewers have distinguished two types of brewer's yeasts. Ale production is performed with top-fermenting yeast strains at a relatively high temperature (18-25°C), and lager beer is fermented with bottom-fermenting strains at lower temperature (8-14°C). Although both species are closely related, important differences, especially with respect to fermentation behaviour and formation of flavours, exist. Generally, bottom-fermenting strains are able to ferment at lower temperatures and are classified on their ability to ferment melibiose. Initially they were named *Saccharomyces carlsbergensis*, or *S. uvarum*. The top-fermenting strains, unable to ferment melibiose, are named *S. cerevisiae*. This distinction between brewer's yeast strains has been abandoned, and all brewer's strains are included in the one species *Saccharomyces cerevisiae* [15].

Differences between the top- and bottom-fermenting strains which are used by the brewer exist e.g. in the behaviour during fermentation. Generally, top-fermenting strains have a more hydrophobic cell wall. They are highly flocculent and form large open flocs. Carbon dioxide bubbles tend to stick and the cell-aggregates are dragged to the surface of the broth. Due to the higher temperatures, fermentation is strong and a large amount of a variety of esters and other flavour components are produced. Flocs formed by lager yeast strains generally have a less open structure. In addition, lager strains generally tend to form higher amounts of sulfur dioxide. However, the more quiet fermentation at lower temperatures leads to lower concentrations of off-flavours, such as diacetyl. The distinction in top and bottom-fermentation has become less clear with the introduction of the cylindroconical vessel in beer production all over the world. Conditions in these tanks are such that also ale yeasts tend to bottom-ferment which makes yeast handling much easier [11, 12].

Beer flavour

Beer flavour consists of over 200 different compounds. The level of individual compounds, as well as the levels relative to each other are important and determine whether or not the flavour is experienced to be pleasant. Often, flavour compounds are essential at very low concentrations, but are regarded off-flavours when levels increase. The major part of the volatile flavours in beer are produced by the yeast. The influence of environmental conditions during fermentation on yeast physiology has a great impact on the final beer flavour [2, 11]. The high number of volatiles which is formed during the main fermentation and maturation can be classified into five groups: alcohols, acids, esters, carbonyls, and sulfur compounds.

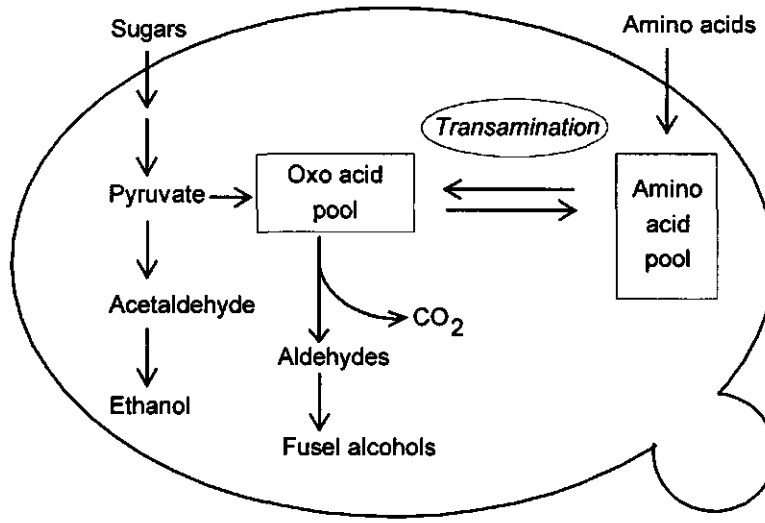

Figure 1 **Metabolic pathways in production of fusel alcohols.**

a. alcohols

Alcohols, of which ethanol (25 - 50 g/l) and glycerol (1.1 - 2.2 g/l) are the predominant ones, reach the highest concentrations [20]. Both are closely linked to glycolysis, the primary metabolic pathway in yeast. Other alcohols, which are generally referred to as higher alcohols or fusel alcohols are: 2- and 3-methylbutanol, 1-propanol, 1-butanol, 2-methylpropanol, and 2-phenylethanol. Fusel alcohols primarily add an alcoholic flavour to beer, however, not all reach their threshold value, generally in the range of 60 to 500 ppm, which thus limits the influence on flavour. Their presence is a result of nitrogen metabolism. Oxo-acids, which are formed by direct transamination of amino acids or by anabolic pathways from fermentable sugar, are decarboxylated to aldehydes which are subsequently reduced to the fusel alcohols by the alcohol dehydrogenases of the yeast (Fig. 1). Factors directly stimulating yeast growth are important. Oxygen, higher temperatures, assimilable nitrogen, and readily metabolizable sugars will increase the levels of the fusel alcohols.

b. acids

Two groups of acids, based on their production pathways, can be identified in beer. The first group consists of organic acids derived from pyruvate or

are intermediates in the tricarboxylic acid cycle [28]. Examples are acetate, pyruvate, succinate, α-ketoglutarate, malate and citrate. These acids generally play a minor role in the flavour of the beer due to low concentrations and high flavour threshold values. The second group is derived from acetyl-CoA in fatty acid synthesis. Medium chain-length (C_6, C_8 and C_{10}) fatty acids produced by this pathway are excreted into the fermentation medium and can account for up to 90% of total beer fatty acids. By chain-elongation, long chain-length fatty acids are synthesized, especially palmitic (C16:0) and stearic acid (C18:0), which are used for lipid synthesis. The unsaturated fatty acids, which are essential building blocks of the membrane lipids, are synthesized from the saturated fatty acids by an oxygen-requiring desaturase (Fig. 2). Concentrations of fatty acids in beer are influenced in much the same way as that of esters, due to the common involvement of acetyl-CoA [29]. Increased concentrations are observed during a maturation with a too high number of yeast cells or if the maturing green beer is not cooled down sufficiently. Under these conditions autolysis of the yeast may occur, resulting in a soapy, caprylic, yeasty or fatty off-flavour.

c. esters

Esters in beer are almost exclusively produced during fermentation. Intracellular enzymes, such as alcohol acetyltransferase (AAT), synthesize the esters by alcoholysis of coenzyme A derivatives [18]. Generally, the most abundant substrates, i.e. ethanol and acetyl-CoA, are coupled, which thus results in ethyl acetate being the most abundant ester (Fig. 2). Due to the link of ester formation with lipid biosynthesis, ester production is low during the growth phase when lipids are produced at a high rate, and increases significantly upon the accumulation of high levels of acetyl-CoA during stationary phase [8]. The final ester concentration is dependent on strain characteristics and fermentation conditions. Generally, factors that lead to more vigorous fermentations, such as high sugar concentrations, increased temperature and low levels of oxygen, will increase ester formation. Based on their composition two groups can be distinguished, viz. the ethyl esters, also referred to as apple esters, and the acetate esters, referred to as banana esters. The low threshold values, 30 ppm for ethyl acetate and 1.6 ppm for isoamyl acetate [20], make that esters have a profound influence on the flavour; controlling their levels is important to obtain a well balanced beer flavour.

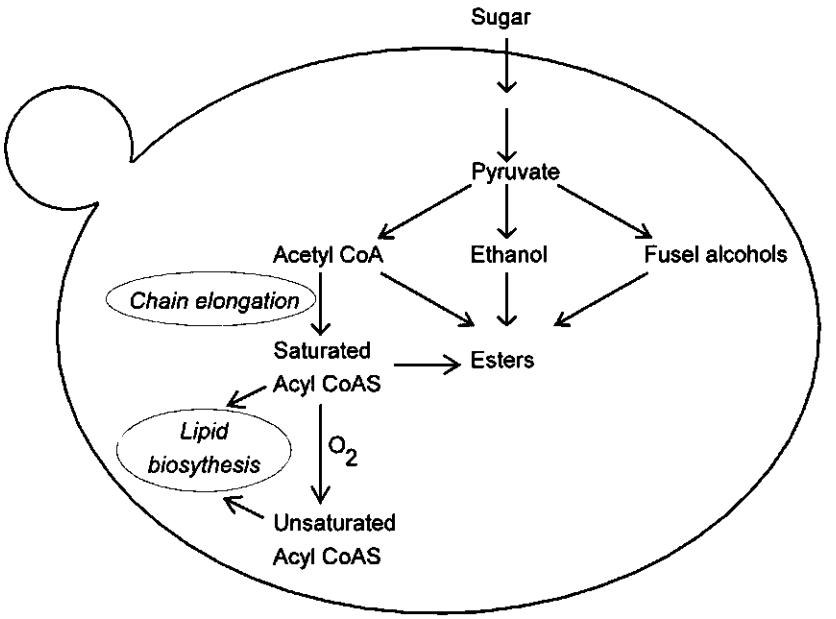

Figure 2 **Metabolic pathways in ester production.**

d. carbonyls

Aldehydes in beer are considered off-flavours, giving the beer an astringent, grassy flavour. During fermentation, most aldehydes present in wort are however reduced to their corresponding higher alcohols, and therefore do not play a significant role. Since the aldehydes are intermediates in yeast metabolism, low amounts may leak from the cell during fermentation. Acetaldehyde is present in the highest concentrations. It is partly driven from the broth by CO_2 evolution, and during maturation levels further decline due to reduction by the alcohol dehydrogenases (ADHs) of the yeast.

Other predominant carbonyls are the vicinal diketones (VDK) diacetyl and 2,3-pentanedione, giving the beer a buttery flavour. These are indirectly formed due to yeast metabolism; during vigorous fermentation elevated levels of oxo-acids diffuse out of the cell (Fig. 1). A subsequent chemical decarboxylation results in the VDK. During maturation, the levels of both diacetyl and 2,3-pentanedione should decrease by intracellular reduction to below 150 ppb, the threshold value of diacetyl.

e. sulfur compounds

The major volatile sulfur compounds, SO_2 and H_2S, are intermediates in the biosynthesis of sulfur-containing amino acids from inorganic sulfur compounds. They accumulate when reduction of sulfate exceeds the formation of the amino acids [11]. Quantities that are formed are highly dependent on the yeast strain used, and on the physiological state of the cells. In addition, the method of filling the fermentor influences concentrations as incremental filling with several batches of wort may lead to a peak in hydrogen sulfide production late in fermentation. Due to its high volatility, H_2S is partly stripped from the fermenting broth by CO_2 evolution. However, concentrations easily reach critical levels since most sulfur compounds have flavour threshold values of less than 10 ppb [20, 21].

Yeast alcohol dehydrogenase

In alcoholic fermentations, a prominent yeast activity is the reduction of aldehydes and formation of alcohols. The enzymes responsible for this are the alcohol dehydrogenases (ADHs), of which *S. cerevisiae* contains several. ADHs present in yeast belong to the group of nicotinamide dinucleotide utilising oxidoreductases (E.C.1.1.1) and catalyse the reaction:

$$\text{alcohol} + NAD^+ \rightleftharpoons \text{aldehyde} + NADH + H^+$$

In *S. cerevisiae*, so far four isoenzymes of ADH have been purified and characterized. All of the isoenzymes are NAD-dependent and their respective structural genes have been identified, cloned, and sequenced. Three of these enzymes are highly homologous and belong to the long chain zinc-dependent ADHs. The primary isoenzyme of *S. cerevisiae* (ADH1) is the fermentative enzyme, which is essential for growth under anaerobic conditions. It preferably uses acetaldehyde as a substrate, is hardly active against longer aldehydes or alcohols [3, 34], and inactive towards branched-chain substrates [10]. The primary function of ADH2 is the oxidation of ethanol to acetaldehyde during gluconeogenesis; expression of the gene is glucose-repressed. Its substrate preference for longer alcohols is higher compared to ADH1 [6, 9], and in addition, glycerol was claimed to be a good substrate [17]. ADH3 is a mitochondrial enzyme of which the function may be linked to the electron transfer chain by production of ethanol during respirative metabolism. ADH1-3 are closely related since all subunits are able to form hybrids [39]. ADH4, which is related to the long chain, iron activated ADHs [30], shows high activity with ethanol, however no activity was found with *n*-butanol [38]. Drewke and Ciriacy [5] described an optimum pH of 8.5 for this enzyme and a quaternary structure which is dimeric. The function of ADH4 is not known [5], but *in vivo* activity could only be detected after overexpression of the structural gene. Recently, evidence for other ADHs was presented. In the yeast genome-sequencing

program, a fifth gene (*adh*5) was detected sharing high homology with the yeast isoenzymes ADH1-3 [7]. In addition, NADP-dependent alcohol dehydrogenases from *Saccharomyces cerevisiae* were described [36, 37].

Production of alcohol-free beer

Production of low and non-alcohol beers is possible by two basically different methods. The first method is based on the production of a high-alcohol beer, of which the alcohol is subsequently removed by different techniques, such as reverse osmosis, dialysis, or evaporation [1, 25, 33]. In contrast, other methods have been developed, based on limited fermentation, resulting in a reduced alcohol content [13, 31, 32]. During limited fermentation, high temperatures (15°C - 20°C) are combined with short fermentation times (0.5 up to 8 hours). However, low temperatures are used as well (0°C - 5°C), often in combination with longer fermentation times (up to 24 hours). In most cases, a high yeast cell concentration is used (> 10^8 cells/ml); thus a thick yeast slurry is mixed with high-gravity wort. A disadvantage of the limited fermentation is that the yeast slurry, which is used for inoculation, may contain a relatively high ethanol concentration (6.5% v/v) [13]. Especially during the short contact times, it is extremely important to have strict control of fermentation, in order to prevent an overshoot in alcohol production. In addition, an inhomogeneous mixture of wort and cells may give problems such as reduced flavour development and cell death.

Immobilized system

To overcome these problems, an immobilized system was developed [4, 16, 35]. Generally, the volume of yeast biomass can increase from about 2% in batch systems up to 15% in immobilized systems, thereby allowing a tenfold reduction in fermentation time [23]. In this system, three aspects are important: (i) The Cultor® carrier, a DEAE-cellulose based granular material, is used in a packed bed reactor, which is operated under down-flow. The yeast cells are bound to the rough surface in a monolayer, and cells are not subject to starvation by substrate limitation (Fig. 3). By packing the carrier particles in a bed reactor, and operating under down-flow, a flexible and a well controllable system is obtained. (ii) A low temperature is used (2°C - 4°C). Yeast growth, which might clog the reactor after long production periods, is suppressed and metabolism is limited. Under these conditions, viability remains high over long periods. (iii) Anaerobic conditions are maintained. The combined stress factors thus suppress yeast growth and sugar metabolism, prevent oxidation of wort lipids to carbonyl off-flavours, and also decrease the risk of microbial contamination [14].

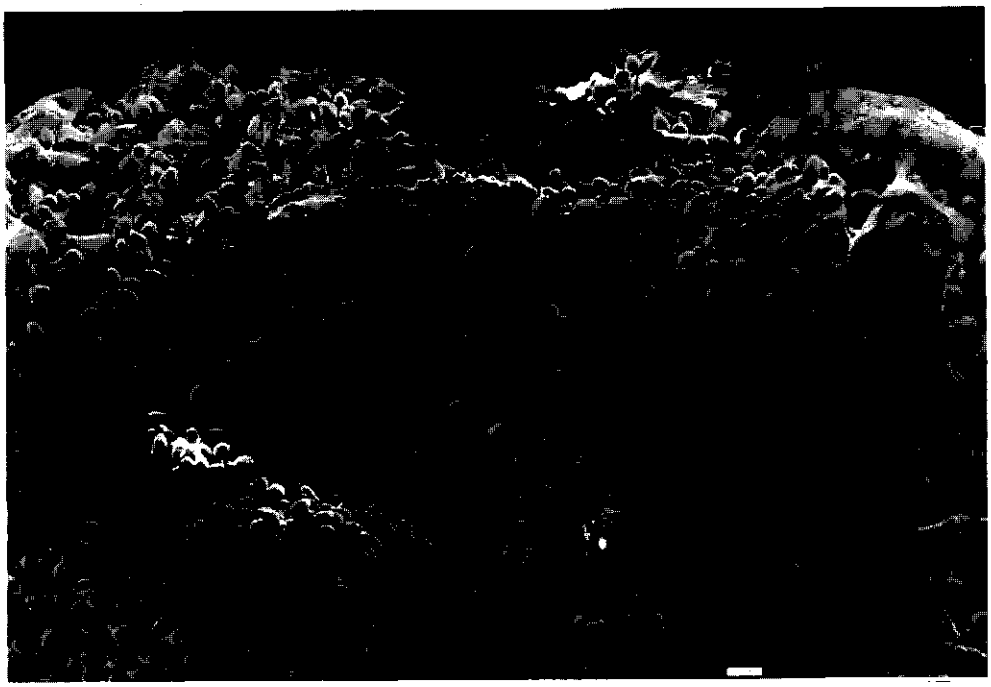

Figure 3 **Scanning-electron micrograph of yeast cells immobilized to a DEAE-cellulose carrier particle. Bar represents 10µm.**

Operating the system

Fermentation is carried out in a packed-bed reactor, operated under down-flow conditions (Fig. 4). The volume of the reactor is 1.5 m^3 and it is usually loaded with 1 m^3 of carrier. In a hydration tank, carrier particles are first hydrated with water at 65°C, sterilized with 2% (w/v) NaOH at 80°C, and neutralized with dilute acid and sterile, carbonated water. When the particles have settled in the bioreactor, wort (12°P) and a yeast suspension are added. After 24 hours of circulation, unbound yeast cells are removed from the reactor and production can start. At first, flow rate and temperature are set at 0.5 m^3/h and 4°C, respectively. Dependent on the overall performance of the reactor, temperature and flow rate are adjusted to values of 2°C and 2 m^3/h. Whenever necessary, production may be stopped for a short period, during which wort is circulated over the reactor at low temperature. After an activation step (rise in temperature, addition of fresh wort and oxygen), production can be resumed. After 5 to 7 months, the entire reactor, including carrier particles, is cleaned and sterilized with 2% NaOH (80°C), and loading and production is started again.

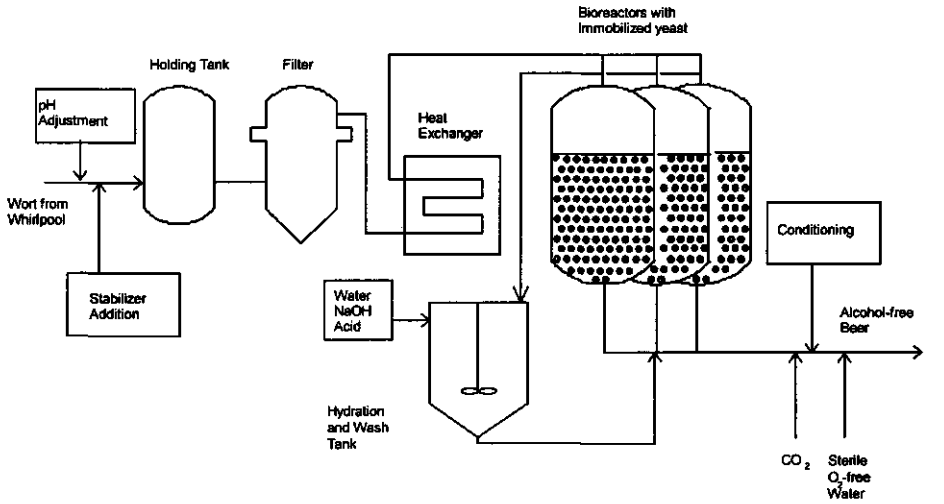

Figure 4 **Production of alcohol-free beer with immobilized yeast.**

Outline of this thesis

Despite clear advantages of immobilized systems in respect to batch systems, few systems have reached the industrial stage. Due to significant changes in cell physiology caused by immobilization processes [19, 22, 26, 27], the translation of well-known batch systems into immobilized systems has proven to be difficult. A novel development in beer production is the production of alcohol-free beer, which is successfully performed with an immobilized system. The aim of this thesis is to shed some light on the reaction of the yeast *S. cerevisiae* towards immobilization under conditions used for production of alcohol-free beer.

First, growth and physiology of the brewer's yeast strain *S. cerevisiae* W34 were characterized (Chapter 2). Since low temperatures are used during alcohol-free beer production, we investigated growth, glycolysis, respiration and biomass production under these conditions.

During alcohol-free beer production, reduction of wort aldehydes is essential for proper flavour formation. This reduction did not correlate with the activity of the known yeast ADHs. We discovered an additional ADH and its purification and partial characterization are reported in Chapter 3.

In Chapter 4 reduction of aldehydes by *S. cerevisiae* and the role of the novel ADH during alcohol-free beer production was further characterized. In addition, a comparison was made between suspended cells and cells immobilized to DEAE-cellulose.

Chapter 5 describes the formation of flavours during production of alcohol-free beer, and relates changes in cell physiology to modifications in flavours during the production process.

In Chapter 6, the influence of temperature on the flocculation behaviour of *S. cerevisiae* was characterized. In addition, we investigated whether flocculation parameters may influence immobilization to DEAE-cellulose.

References

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GROWTH AND PHYSIOLOGY OF *SACCHAROMYCES CEREVISIAE* AT LOW TEMPERATURES

Abstract

In the present study we investigated the influence of temperature on the physiology of a brewer's yeast strain of *Saccharomyces cerevisiae* var. *uvarum*. Growth occurred in a temperature range of -2°C to 30°C with long lag times at low temperatures. Subsequently, sugar metabolism was analyzed with cells grown at 25°C or at 4°C. Similar rates of metabolism were found, although high or low temperature grown cells were subjected to a downward or upward temperature shift to the assay temperature of 4°C or 25°C. Furthermore, by fluorescent labeling with the mitochondrial dye DASPMI, active mitochondria were observed in cells grown at 25°C or at 4°C. However, the latter type lacked substrate-induced respiration upon addition of fermentable sugar or ethanol. In addition, an increase in glycerol formation, and a decrease in biomass yield on glucose was observed in cells grown at 4°C. Calculating the yield of ATP on substrate, it appeared that at 25°C but not at 4°C, respiration is needed to reach the theoretical yield.

The changes in yield coincided with an increase in *in vitro* hexokinase activity in low temperature grown cells, which may be linked to an increased glucose repression. Influence of low growth temperatures on yeast physiology is being discussed

Submitted for publication by M. van Iersel, F. Rombouts and T. Abee.

Introduction

Biotechnological fermentation processes are mainly performed at higher temperatures (> 25°C), owing to the fact that production proceeds faster and at lower costs. However, if formation of flavor components is of importance, lower temperatures are used as most components are very volatile [4]. Examples include the production of lager beer (7-12°C), low-alcohol beer (0-4°C) and cider fermentation (10°C) [4, 24, 25].

Both cell composition and metabolism of microorganisms are affected by cultivation temperature [30]. Generally, cellular fatty acid composition shows a higher ratio of unsaturated to saturated and short-chain to long-chain fatty acids, and higher concentrations of RNA and protein have been reported at low temperatures [19]. Cold-shock proteins, which facilitate translation processes by minimizing secondary folding of RNA, are transiently induced during cold adaptation [12, 23].

Cell metabolism is affected by temperature modulation of enzyme kinetics, which changes the end products and their concentrations. Higher fermentation temperatures are e.g. known to increase formation of esters and decrease the rate of acid production [4, 7]. Low temperatures on the other hand, decrease formation of α-acetolactate [25], and increase fusel oil production [10].

Since the production of flavors is affected by temperature, fluctuations in temperature are preferably avoided during fermentations. During production of alcohol-free beer, a limited fermentation using immobilized yeasts, temperature is kept low (0 to 4°C) to suppress yeast growth [25]. In this article we describe the influence of such low temperatures on a brewer's yeast strain of *S. cerevisiae*. Growth, fermentation, glycolytic activity, respiration and mitochondrial morphology were compared between high and low temperature grown cells.

Materials & Methods

Strain and cultivation conditions

Saccharomyces cerevisiae var. *uvarum* strain W34 was obtained from the Institute of Weihenstephan, University of Munich, Germany. *S. cerevisiae* W34 was grown in MYGP containing malt extract (3 g/l), yeast extract (3 g/l), glucose (10 g/l) and mycological peptone (5 g/l). Aerobic incubations were performed in a temperature controlled rotary shaker operated at 170 rpm. Growth was followed by plating on MYGP agar and counting the number of colonies (cfu). Growth rates were calculated by fitting the curves with a modified Gompertz equation according to Zwietering *et al.* [31]

Preparation of cell extracts

Yeast cells were harvested at the end of logarithmic phase, prior to the onset of oxidative growth. After washing, cells were concentrated to approx. 30 g dry weight l^{-1}. Cells were subsequently broken by vortexing them with glass beads [25].

Enzyme assays

Spectrophotometric assays were carried out with a Beckman DU-60 spectrophotometer, supplied with a Kinetics Soft-Pac module and temperature incubator (Beckman, Fullerton, USA). NAD(P)H absorption was measured at 340 nm. With all assays, rates were linearly proportional to the amount of extract added. One unit is defined as the conversion of 1 µmol NAD(P)H per min.

Glucokinase: 50 mM imidazole·HCl buffer, pH 7.6, 10 mM $MgCl_2$, 1 mM $NADP^+$, 50 U glucose-6P dehydrogenase, 10 mM D-glucose. The reaction was started with 1 mM ATP.

Hexokinase (HEX): 50 mM imidazole·HCl buffer, pH 7.6, 10 mM $MgCl_2$, 1 mM $NADP^+$, 50 U glucose-6P dehydrogenase, 10 U glucose-6P isomerase, 10 mM D-glucose. The reaction was started with 1 mM ATP.

Alcohol dehydrogenase (ADH): 50 mM glycine KOH, pH 9.5, 1 mM NAD^+. The reaction was started with 100 mM ethanol.

Pyruvate decarboxylase (PDC): 40 mM imidazole·HCl, pH 6.5, 5 mM $MgCl_2$, 0.15 mM NADPH, 0.2 mM thiamin pyrophosphate, 88 U alcohol dehydrogenase. The reaction was started with 50 mM pyruvate.

Malate dehydrogenase (MDH): 100 mM KP_i, pH 7.5, 0.15 mM NADH. The reaction was started with 1 mM oxaloacetate.

NADP-linked isocitrate dehydrogenase: 100 mM KP_i, pH 7.0, 5 mM $MgCl_2$, 0.4 mM $NADP^+$. The reaction was started with 1 mM isocitrate.

NAD-linked isocitrate dehydrogenase: 100 mM KP_i, pH 7.0, 2.5 mM $MgCl_2$, 0.5 mM AMP, 0.4 mM NAD^+. The reaction was started with 5 mM isocitrate.

ATP measurements

The ATP concentration was measured with a Lumac/3M biocounter M2010 using the luciferine-luciferase enzyme assay (Lumac, Landgraaf, The Netherlands). The enzyme, Lumit-PM, was reconstituted in Lumac buffer, according to the manufacturers indications. ATP was extracted from the cells using DMSO; 20 µl cell sample was added to 80 µl DMSO, mixed and diluted with 5 ml Nanopure-filtered water. For the analysis, 100 µl enzyme was added to 200 µl sample.

Respiration measurements

Oxygen consumption was determined using a polarographic oxygen electrode (Clark type) in a stirred vessel (Biological Oxygen Monitor, Yellow Springs, USA). Cells were diluted in air saturated MES-buffer (0.1 M, pH 5.0). After determination of endogenous respiration, sugar or ethanol were added to a final concentration of 20 mM.

Fluorescent labeling

Cells were labeled with the mitochondrial specific dye, 2-(4-(dimethylamino) styryl)-N-methylpyridinium iodide (DASPMI) obtained from Molecular Probes (Leiden, The Netherlands). DASPMI was added to a cell suspension at 40 µM (final concentration) in 0.1

M Tris pH 8.0. Cells were incubated for 15 min in the presence of 50 mM glucose and analyzed with a fluorescence microscope (Zeiss Axioskop, Oberkochen, Germany).

Analytical methods

Concentrations of sugars and fermentation products were determined in samples obtained by filtration over a 0.45 µm membrane filter. Sugars were determined by high-performance anion-exchange chromatography (HPAEC) with pulsed-amperometric detection. Chromatography was performed using a Dionex Bio-LC system equipped with a Carbopac PA 100 column, 4 x 250 mm (Dionex, Sunnyvale, USA). Separation was achieved with a combined gradient of three eluents, viz. 0.1 M NaOH, 1 M sodium acetate in 0.1 M NaOH, and distilled water. Fermentation products, i.e. ethanol, glycerol and acetic acid, were determined by HPLC (SpectraPhysics, San Jose, USA) using an Aminex HPX-87H column (300 x 7.8 mm) from Bio-Rad (Veenendaal, The Netherlands) and refraction index detector, Shodex RIse-61 (Showa Denko, Tokyo). Elution was performed with 50 mM H_2SO_4 at 40°C and a flow rate of 0.6 ml min^{-1}. All eluents were prepared with distilled water that had been filtered with a Nanopure-system.
Protein concentrations were determined according to the method of Lowry *et al.* [14]. Optical density (OD) was measured at 600 nm and standard curves of OD vs. dry weights (DW) were prepared for exponentially growing cells at both 25°C and 4°C. DW was determined by filtration over predried and preweighed nitrocellulose filters (0.45 µm). Filters were dried at 80°C.

Results

Growth

The ability of *Saccharomyces cerevisiae* to grow under aerobic conditions was tested over a wide range of temperatures (Fig.1). Growth rates decreased at the low temperatures, with the corresponding lag phases increasing significantly (Fig. 1A). The relationship between growth rate and temperature can be described with the Ratkowsky equation [17]. Calculating the minimal growth temperature, it appeared that this yeast has the ability to grow at temperatures down to -2°C. In addition, the number of cfu's which were reached in stationary phase decreased substantially at temperatures below 6°C (Fig. 1B).

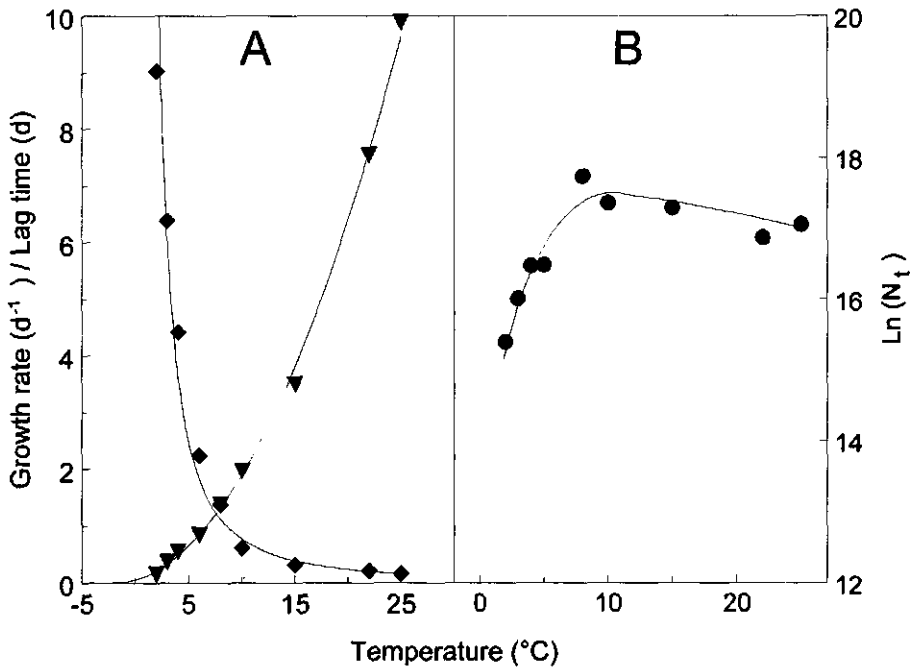

Figure 1. **Aerobic growth of *S. cerevisiae* on a complex medium (MYGP). (A) Growth rate (▼) and lag time (◆) and (B) asymptote of growth (●) at different temperatures.**

Sugar fermentation

To analyze whether the increase in lag time was caused by inhibition of sugar metabolism, we investigated the ability of the cells to ferment glucose or fructose at low temperature. Cells were grown at 4°C and 25°C and uptake of glucose was determined at both temperatures (Fig. 2). Uptake and fermentation of glucose by the yeast cells started immediately, even when 25°C grown cells were tested at low temperature. Similar results were obtained with fructose and maltose (data not shown). Generally, transport rates of glucose or fructose at 4°C were approximately 10% of the rates at 25°C.

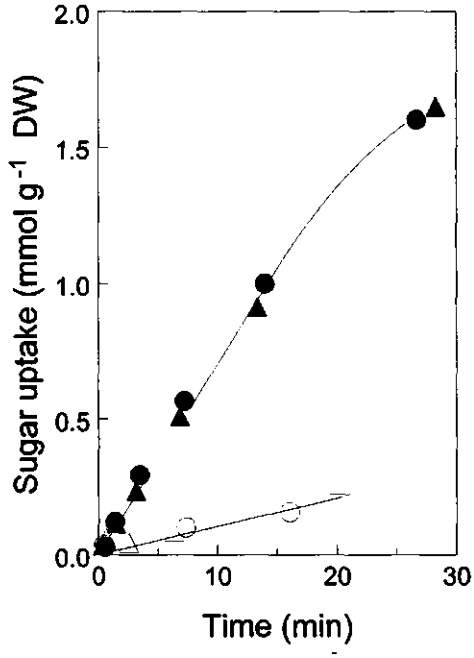

Figure 2. **Uptake of glucose (20 mM) by *S. cerevisiae*. Uptake was measured at 4°C (open symbols) and 25°C (closed symbols). Cells were grown at 4°C (●,○) and at 25°C (▲,△) and harvested in the exponential phase.**

Furthermore, uptake of different fermentable sugars at various concentrations was determined (Table 1). Rates of uptake of glucose and fructose were in the same range as that of maltose in maltose-grown cells. A further increase in sugar concentration, up to 50 mM, did not reveal differences in sugar metabolism between cells grown at 4°C and 25°C (data not shown).

In addition, ATP measurements of high and low temperature grown cells, assayed at both 4°C and 25°C, showed an immediate increase in intracellular ATP concentrations upon addition of sugar to these cells (data not shown).

Table 1. **Effect of temperature on sugar metabolism of *S. cerevisiae* grown at 4°C or 25°C. Rates were measured at both 25°C and 4°C with 20 mM or 1 mM of each sugar.**

Substrate	Assay temperature	Sugar concentration			
		20 mM		1 mM	
		Growth temperature			
		25°C	4°C	25°C	4°C
Maltose	25°C	63 [1]	43	4.6	n.d. [2]
Glucose		104	86	28	12
Fructose		68	71	11	6.4
Maltose	4°C	7.3	6.5	0.6	n.d.
Glucose		11	7.0	2.8	2.5
Fructose		8.2	8.3	1.6	1.1

[1] Sugar consumption rates expressed as μmol min^{-1} g^{-1} DW
[2] n.d.: not determined

To determine the flux of sugar to different products, concentrations of ethanol, glycerol and acetic acid were determined at high and at low temperature during the fermentation assays (Table 2). It appeared that the formation rates of ethanol and glycerol were higher with cells grown at 4°C than with cells grown at 25°C.

Table 2. **Fermentation rates at high (25°C) and low temperature (4°C). Rates of product formation were determined after addition of 20 mM glucose to cells grown at 25°C and 4°C.**

Assay Temperature	Growth Temperature	Ethanol	Acetate	Glycerol
		Production rates (μmol min^{-1} g^{-1} DW)		
25°C	25°C	80.9	1.00	0.93
	4°C	139	0.56	5.81
4°C	25°C	10.0	0.27	0.32
	4°C	10.9	0.49	0.42

Enzyme activity

Activities of glycolytic enzymes were determined in cell extract (CE) of *S. cerevisiae* W34. Assays were performed at high (30°C) as well as low

temperature (5°C), and an approximate fourfold decrease was observed due to assay temperature (Table 3). This is in accordance with theoretical calculations using the Arrhenius equation [19]. Furthermore, activities were determined in CE from cells grown at 4°C or at 25°C. With the activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) only slight differences were observed. However, glucokinase and hexokinase (HEX) activities increased approximately 7-fold in 4°C grown cells, compared to 25°C grown cells (Table 3).

Table 3. **Enzyme activities of *S. cerevisiae*. Cells were grown aerobically at 25°C or 4°C. Activities were linearly proportional to the amount of protein and time.**

Assay temperature		Growth temperature 25°C	4°C
		Enzyme activity (U mg^{-1} protein)	
30°C	Glucokinase	0.36	2.5
	Hexokinase	0.49	3.2
	Pyruvate decarboxylase	0.52	0.20
	Alcohol dehydrogenase	0.32	0.54
5°C	Glucokinase	0.08	0.36
	Hexokinase	0.11	0.73
	Pyruvate decarboxylase	0.12	0.05
	Alcohol dehydrogenase	0.06	0.10

In addition to glycolytic enzymes, enzymes of the tricarboxylic acid cycle, such as malate dehydrogenase (MDH), and isocitrate dehydrogenase (IDH) were assayed. Here, activities of MDH, and NAD and NADP-dependent IDH were determined at 0.74, 0.021 and 0.023 U mg^{-1} protein respectively, for both high and low temperature grown cells.

Respiration

To determine the influence of temperature on respiration, both high and low temperature grown cells were suspended in buffer of 25°C, and the decrease in oxygen was measured using a Clark-type electrode. It appeared that cells grown at 4°C or 25°C had a similar level of endogenous respiration. However, upon addition of fermentable sugar or ethanol, respiration increased significantly in 25°C grown cells, whereas with 4°C cells no such increase was observed. A similar trend was observed when the substrate-induced respiration was assayed at 4°C (Table 4).

Table 4. **Respiration of *S. cerevisiae* grown at 4°C or 25°C and assayed at 4°C and 25°C. Respiration was measured with a Clark-type electrode before and after addition of glucose, fructose or ethanol (20 mM).**

Assay temperature		Growth temperature 25°C	4°C
		Oxygen consumption (μmol min^{-1} g^{-1} DW)	
25°C	No addition	10 ± 1.2	11 ± 0.8
	Glucose	28 ± 1.5	11 ± 0.8
	Fructose	30 ± 1.0	11 ± 0.8
	Ethanol	31 ± 2.2	11 ± 0.8
4°C	No addition	1.3 ± 0.4	2.4 ± 0.6
	Glucose	3.7 ± 0.6	2.4 ± 0.6
	Fructose	3.7 ± 0.5	2.4 ± 0.6
	Ethanol	4.9 ± 0.9	2.6 ± 0.6

Due to the substrate-induced respiration in 25°C grown cells, it may be expected that the biomass yield on glucose is higher in those cells compared to that of 4°C grown cells. This was indeed observed. Ratios of biomass production per glucose consumption, determined from the increase in OD and decrease in glucose during the exponential growth phase, were calculated at 34 and 21 g DW M^{-1} glucose for 25°C and 4°C grown cells, respectively.

Mitochondrial morphology

Absence of substrate-induced respiration in cells grown at 4°C, may be caused by formation of respiratory-deficient mutants. Therefore, the morphology of DASPMI-stained mitochondria was analyzed using fluorescence microscopy. Accumulation of styryl dyes in lipid membranes is a function of membrane potential and therefore the dye preferentially accumulates in active mitochondria [8, 16]. In 4°C grown cells, brightly fluorescing mitochondria were observed, indicating the presence of membrane potential and actively respiring mitochondria (Fig. 3). The morphology of these mitochondria resembled that of glucose-repressed cells in that they existed of large, ramified structures. In contrast, small, bud-like mitochondria are generally observed in glucose-derepressed cells [21, 27]. In cells grown at 25°C, a low number of these bud-like mitochondria was observed in addition to the large ramified structures (data not shown).

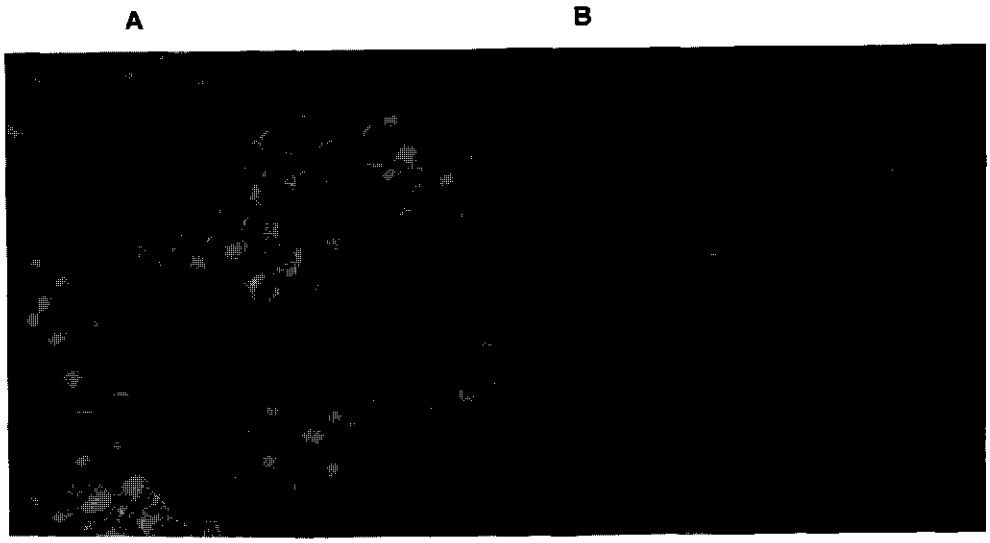

Figure 3. **Fluorescent staining of mitochondria with DASPMI. Cells were grown at 4°C and staining was performed according to Materials and Methods. (A) Phasecontrast image (B) Fluorescent image.**

Discussion

Influence of temperature on growth and fermentation

Saccharomyces cerevisiae was able to grow at a wide temperature range. A minimal growth temperature of -2°C was calculated according to the Ratkowsky equation. In addition, a significant increase in lag time was observed at the lower temperature range (Fig. 1). The increase in lag time did not correspond with the fermentation assays, cells took up glucose and metabolized it instantly whether or not they were subjected to a temperature shock, such as 25°C grown cells analyzed at 4°C (Fig. 2). In addition, an immediate increase in total ATP concentration was observed upon glucose addition. Apparently, the increase in lag time at low temperatures cannot be explained by a defect in substrate transport and metabolism, or a lack of sufficient energy. More likely, protein synthesis had to be restored after a temperature down-shock and this cold shock response caused the increase in lag time. In case of large temperature shifts, the cold shock response is more pronounced and cells need more time to adjust [20, 23].

Respiration and growth yield

In Table 4 it was shown that substrate-induced respiration is absent in 4°C grown cells of *S. cerevisiae* W34. No literature is available about the respiration of *S. cerevisiae* at low temperature. With other yeast species such a lack of substrate-induced respiration was not observed. In mesophilic, as well as in psychrophilic *Candida* spp., Baxter and Gibbons [2] reported a steady decline in respiratory capacity with decreasing temperatures; even at 0°C respiration was observed.

The absence of substrate-induced respiration in cells grown at 4°C coincided with an increase in fermentation products compared to 25°C-grown cells. In addition, we observed a significant decrease in yield of biomass on glucose during exponential growth, which resulted in lower amounts of cfu's reached in stationary phase (Fig.1). The decrease in yield could not be explained by an increase in maintenance energy at low temperature since generally maintenance can be neglected in batch cultures where the energy source is non-limiting [13, 15]. The absence of substrate-induced respiration may offer an explanation. If we assume a Y_{ATP} (energy requirement for biomass production) of 10.5 g DW mol^{-1} ATP [1, 9, 26], the ATP yield on glucose is 3.3 mol ATP mol^{-1} glucose for 25°C grown cells during exponential growth. For cells grown at 4°C, a value of 2.0 mol ATP mol^{-1} glucose is calculated, which equals the net amount of ATP gained in the dissimilation of glucose to ethanol. The ATP yield on glucose in 25°C grown cells however, exceeds this value. If we assume a net production of 36 moles ATP in the TCA, approximately 5% of the glucose needs to be directed towards the TCA to reach a yield of 3.3 mol ATP mol^{-1} glucose. This indicates that in 25°C grown cells, a low amount of ATP is derived from respiration, whereas in 4°C grown cells, glycolysis satisfies to reach the appropriate ATP-yield.

Mitochondrial morphology

Literature about the influence of temperature on mitochondrial structure and inheritance is limited to the higher temperature range. Mitochondrial inheritance was reported to be irregular at temperatures near the maximal temperature of growth, but good inheritance was observed when cells were incubated at temperatures slightly below or near the optimal growth temperature [21]. Extensive literature is available on the effect of growth phase and media composition on mitochondrial morphology [21]. The amount as well as the total volume of the mitochondria is reported to increase significantly upon exhaustion of the fermentable sugar, i.e. when the metabolism of the cell changes from a respirofermentative state to respiration derepressed cells. Mitochondria break up into smaller parts and the initial volume of 3 to 4% increases to approximately 10 to 12% of total cell volume [21].

In the yeast *S. cerevisiae* W34 we observed only small differences in mitochondrial morphology between cells grown at high and at low temperature. The overall morphology of the mitochondria under both conditions resembled that of glucose-repressed cells. In cells grown at 25°C, a low number of bud-shaped mitochondria was observed, which may indicate an increase in surface area.

Glucose repression at low temperature

When growing on fermentable sugars, cells of *S. cerevisiae* exhibit glucose repression. Enzymes involved in respiration, the TCA-cycle and gluconeogenesis are inhibited whereas the activity of the glycolysis is increased [3, 6, 22]. Cells of strain W34, growing on glucose at low or high temperature, were glucose repressed, as indicated by the low activities of ADH, MDH and NAD(P)-dependent IDH, mitochondrial structure and a low respiration activity [6, 11, 21]. In the case of growth at low temperature, some of these characteristics were even more pronounced, substrate-induced respiration was absent and growth yield was severely reduced. In addition, in cells grown at 4°C, hexokinase activity was increased 7-fold compared to cells grown at 25°C. From literature it is known that the glucose phosphorylation capacity is in excess of glucose flux [5, 28], and hexokinase is claimed to be involved in glucose repression [18, 29]. The significant increase in HEX activity may thus indicate that cells growing at low temperature were subjected to a higher level of glucose repression, compared to cells grown at 25°C.

Conclusion

We report that cells of *S. cerevisiae* strain W34 were observed to be able to grow at temperatures as low as -2°C. Although mitochondrial morphology indicated active mitochondria, substrate-induced respiration was absent in cells grown at low temperatures. This resulted in a substantial reduction of the growth yield. Coinciding with these changes, we observed an increase in hexokinase at low growth temperatures, which may be linked to an increased glucose repression.

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PURIFICATION AND CHARACTERIZATION OF A NOVEL NADP-DEPENDENT BRANCHED-CHAIN ALCOHOL DEHYDROGENASE FROM *SACCHAROMYCES CEREVISIAE*

Abstract

An NADP-dependent branched-chain alcohol dehydrogenase was purified from *Saccharomyces cerevisiae* var. *uvarum* grown under anaerobic conditions. Its quaternary structure is monomeric with a molecular mass of 37 kDa and a pI of 5.9. A possible role of the enzyme in flavour production during alcoholic fermentations is discussed.

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Yeast alcohol dehydrogenase

A large number of different compounds contribute to the flavour of alcoholic beverages. Among these, aldehydes and alcohols have a high impact, caused by low flavour threshold values and high concentrations, respectively. Generally, aldehydes are considered off-flavours, but fortunately their presence is limited due to conversion by alcohol dehydrogenases (ADHs) of *Saccharomyces cerevisiae*. Alcohol dehydrogenases present in yeast belong to the group of nicotinamide dinucleotide utilizing oxidoreductases (E.C.1.1.1). In *S. cerevisiae*, so far four isoenzymes of ADH have been purified and characterized. All of the isoenzymes are NAD-dependent and their respective structural genes have been cloned and sequenced [1, 7, 9, 13, 14].

In alcoholic fermentations, activity of the ADHs is not limited to acetaldehyde. Several so-called Strecker aldehydes, formed by transamination and subsequent decarboxylation of amino acids, are reduced to fusel alcohols. The final levels of these alcohols are determined by environmental factors, e.g. oxygen, temperature, substrate, and amino acids, as well as strain characteristics [11]. During production of alcohol-free beer, anaerobic conditions are used and temperature is kept low, suppressing yeast growth and fermentation [16]. ADH1 was found not to be involved in reduction of branched chain and higher aldehydes since no correlation was found between reduction of these compounds, and the activity of this enzyme [2]. Here, we report the purification and characterization of a novel NADP-dependent branched-chain alcohol dehydrogenase (bcADH) from a brewer's yeast strain of *Saccharomyces cerevisiae*, which is expressed under anaerobic growth conditions.

Expression of ADH activity

Saccharomyces cerevisiae var. *uvarum* W34 was obtained from the Institute of Weihenstephan, Technical University of Munich, Germany. Cells were grown in MYGP (malt extract 3 g l^{-1}, yeast extract, 3 g l^{-1}, mycological peptone, 5 g l^{-1} and glucose 10 g l^{-1}) at 25°C on a rotary incubator (170 rpm) and ergosterol and Tween-80 were added under anaerobic conditions [17]. In cell extract (CE) from strain W34 grown under anaerobic conditions, a significant increase was observed in NADPH-dependent aldehyde reductase activity, compared to CE obtained from cells grown under aerobic conditions [12]. CE of late-exponential aerobic and anaerobic cells were therefore separated on an FPLC Mono-Q column (HR 5/5), using a linear gradient of 0-200 mM NaCl in 20 mM BisTris, pH 7, 0.1 mM $MgCl_2$, 2 mM DTT (Fig. 1). Measuring the reduction of 3-methylbutanal with CE of aerobically grown cells, we found one activity peak with each coenzyme (Fig. 1A). In cells grown under anaerobic conditions however, two activity peaks with each

coenzyme were found; the first eluted at approximately 50 mM NaCl, and the second at 100 mM NaCl (Fig. 1B).

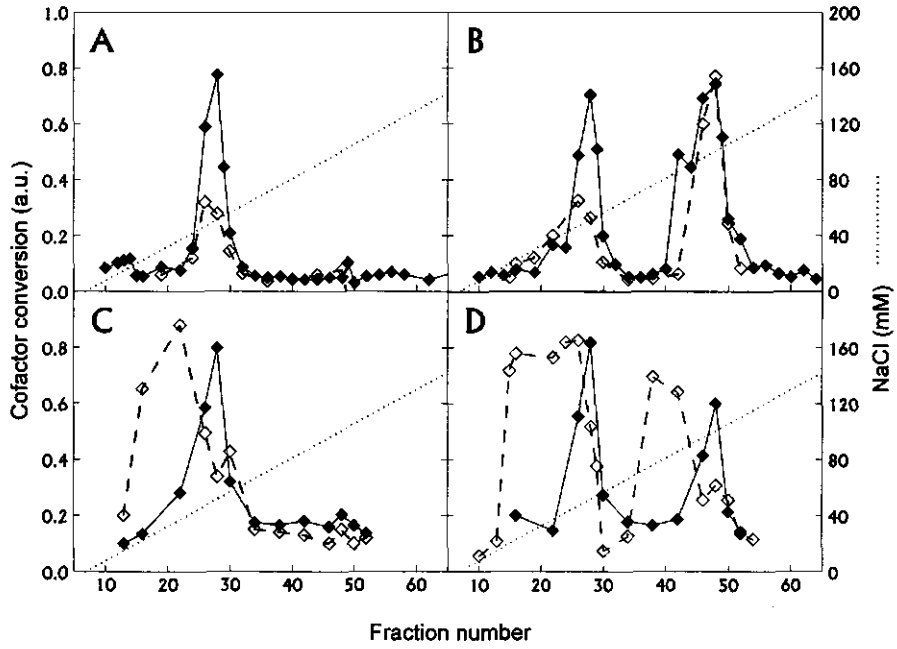

Figure 1. **Reduction of 3-methylbutanal (A, B) or hexanal (C, D) with fractions (1 ml) of CE from *S. cerevisiae* strain W34 obtained after ion exchange chromatography on FPLC Mono-Q HR 5/5. CE was obtained from cells grown under aerobic conditions (A, C) or anaerobic conditions (B, D). Oxidation of NADPH (♦) or NADH (◊) was analyzed fluorimetrically.**

Reduction of hexanal was also tested (Fig. 1C and 1D). For each cofactor, one peak was found in CE of aerobic cells (Fig. 1C) and in CE of anaerobic cells for each coenzyme two peaks appeared (Fig. 1D). In contrast to the activity with 3-methylbutanal, the activity peaks of NADH-driven reduction with hexanal did not coincide with peaks of NADPH-driven reduction. Similar results were obtained with acetaldehyde (data not shown).

These results suggest that different types of alcohol dehydrogenase were present in the CE of the yeast depending on the growth conditions. Apparently, the increased aldehyde reductase activity in CE of anaerobically grown cells was related to the appearance of the additional peak in the Mono-Q chromatogram showing 3-methylbutanal reductase activity. Subsequently, we

aimed to purify this enzyme since it may play a role in flavour formation under anaerobic conditions i.e. during alcoholic fermentations.

Enzyme purification

Cells were broken by four passages through a French Press (600 kg cm^{-2}). After removing cell debris by centrifugation (30,000 x g, 40 min), the ADH present in the second activity peak of anaerobic CE (at 100 mM NaCl) was purified in a three step procedure (Table 1). During purification, samples were analyzed for enzyme activity measuring decrease in NAD(P)H fluorescence. Already the first step (Q-Sepharose column calibrated with 20 mM BisTris, pH 7, 0.1 mM $MgCl_2$, 2 mM DTT) resulted in a high purification factor, owing to the relatively high concentration of NaCl at which this ADH eluted.

Table 1. Purification of ADH expressed under anaerobic conditions in *S. cerevisiae* strain W34.

Purification step	Total protein (mg)	Total activity [a] (U)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification fold (-)
Cell Extract	10100	159 (366)[b]	0.016 (0.036)	100 (100)	1 (1)
Q-Sepharose	147	150 (226)	1.02 (1.54)	94 (62)	65 (43)
Phenyl Sepharose	60	112 (133)	1.86 (2.21)	70 (36)	118 (61)
Red Agarose	0.46	28 (43)	61.6 (93.9)	17 (12)	3920 (2593)

[a] Reduction of 3-methylbutanal was measured at pH 6.7 with NADPH as coenzyme.
[b] Numbers in parenthesis are the activity with the coenzyme NADH.

Subsequently, $(NH_4)_2SO_4$ was added to a final concentration of 1 M and the sample was applied to a Phenyl Sepharose column (2.6 cm x 10 cm, Pharmacia, Uppsala, Sweden). Elution occurred in BT-buffer without salt. After dialysis against 20 mM BisTris, pH 8.0, 0.5 mM DTT and 5 mM $MgCl_2$, sample was applied to a Procion Red dye affinity column (Red Agarose, Amicon Inc., Beverly, USA), which was selected based on its capacity to bind to a wide range of NADP-dependent enzymes. The enzyme was eluted with 0.2 mM $NADP^+$ in the same buffer, and cofactor was removed by three successive dilution and ultrafiltration steps. With this column, a high degree of purification was obtained (Table 1). No rationale was found for the low yield of this step, since reductase activity was absent in fractions obtained during or shortly after loading of the column, nor after washing the affinity column with a high concentration of salt (1 M NaCl). Nevertheless, the high purification fold indicates that the total amount of enzyme present in the CE was low; it is estimated at approximately 0.03%. The ratio of the activities with NADH over NADPH on 3-methylbutanal remained more or less

constant during the purification (Table 1), which suggests that one specific type of ADH was being purified.

Structural properties

Estimation of the molecular mass of the purified enzyme by SDS-PAGE (Fig. 2) as well as by gel filtration on an FPLC Superose-12 column HR-10/30 (data not shown) revealed an apparent molecular mass of 37 kDa. Therefore, it is presumed that no association of subunits occurs, in contrast to other yeast isoenzymes, which function as di- or tetramers [1, 6, 13]. Isoelectric focusing of the purified enzyme revealed a single band with a pI of 5.9. In order to determine the N-terminal amino acid sequence, 300 pmole purified protein was blotted onto PVDF-membrane. However, the results of the Edman degradation were negative, presumably due to blocking of the N-terminus.

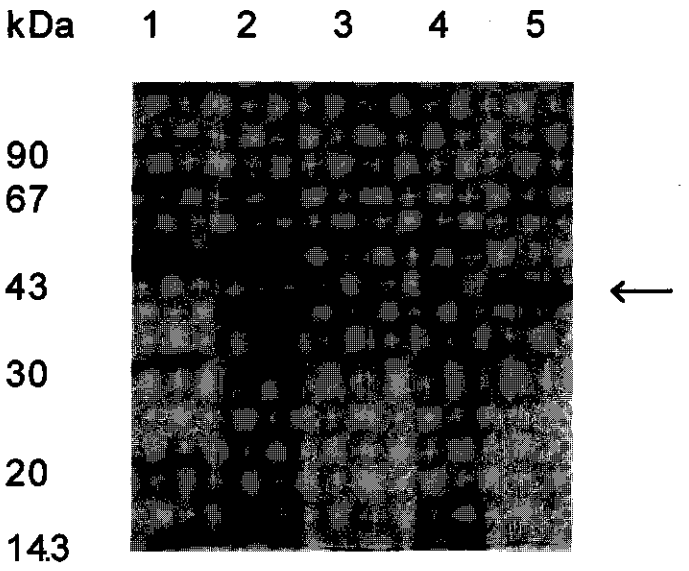

Figure 2. **Analysis of enzyme purification by SDS-PAGE. Lanes: 1, Marker; 2, CE; 3, Q-Sepharose pool; 4, Phenyl Sepharose pool; 5, Red Agarose pool. Each lane contains approximately 3 µg protein, except lane 2 to which approx. 10 µg was loaded.**

Coenzyme specificity

To determine the coenzyme specificity in more detail, Michaelis constants for both NADH and NADPH were determined in the reduction with 3-methylbutanal. Kinetic parameters were determined using a Hitachi U-3000 Spectrometer (Hitachi Ltd., Tokyo, Japan). The affinity of the enzyme was highest for the phosphorylated coenzyme (K'_m = 20 µM), whereas that for NADH was approximately 80-fold lower (K'_m = 1.7 mM). In addition, reduction experiments were performed in the presence of both reduced and oxidized coenzymes. Hardly any effect on the oxidation of NADPH (0.15 mM) was observed when an excess of NAD^+ or $NADP^+$ were added. However, similar concentrations of NAD^+ or $NADP^+$ showed a severe effect on a reduction assay with NADH (0.15 mM); a 6.7-fold excess of $NADP^+$ completely inhibited this reaction, suggesting competition for the same binding site.

Substrate specificity

Substrate specificity of the purified ADH was elucidated by measuring the rates of aldehyde reduction and alcohol oxidation. Aldehydes or alcohols were solubilized in buffer by sonication, using a method adapted from Wales and Fewson [18]. Reduction assays were performed at 30°C in a buffer containing 30 mM of each MES, MOPS and Tris (MMT), pH 6.7, *I*=0.03 M, 0.15 mM NAD(P)H, and enzyme. Oxidation of alcohols was performed in 20 mM CHES, pH 10 and 1 mM $NAD(P)^+$. Reactions were started by addition of 10 mM aliphatic substrate.

Kinetic measurements revealed substrate inhibition at high concentrations of aldehydes. In order to calculate kinetic parameters, a modified Michaelis-Menten equation was used as described by Dixon and Webb [5]:

$$v = V'_{max} / (1 + [K'_m / S] + [S / K'_i]) \qquad [1]$$

where v is the initial rate (s^{-1}) at substrate concentration S (mM), K'_m is the apparent Michaelis-Menten constant, K'_i the apparent substrate inhibition constant and V'_{max} the apparent reaction rate at saturating substrate concentration. Application of this equation on a two substrate reaction is allowed at saturating coenzyme concentrations. When fitting the equation to the observed data, excellent correlation was found (generally $r^2 > 0.99$).

Table 2 shows that both K'_m and K'_i decreased with increasing chain length. The catalytic efficiency (k'_{cat} / K'_m) was highest with hexanal, whereas with 3-methylbutanal, the highest ratio of K'_m/K'_i was observed, indicating that this is the better substrate. Kinetic analysis of alcohol oxidation (hexanol and *t*-2-hexenol) revealed a significantly lower catalytic efficiency of the enzyme. The enzyme was also highly active towards aromatic aldehydes such as benzaldehyde, *p*-anisaldehyde and furaldehyde, which had 20 to 50% of the activity observed with

3-methylbutanal. No reduction was observed with ketones, pyruvate and glucose, and with vicinal diketones such as diacetyl and 2,3-pentanedione only a very low activity was observed, approximately 5% of the activity with 3-methylbutanal (data not shown).

Table 2. Kinetic parameters for the purified bcADH from *S. cerevisiae* W34. [a]

	NADPH				NADH			
	K'_m (mM)	K'_i (mM)	k'_{cat} (s^{-1})	k'_{cat}/K'_m ($M^{-1}s^{-1}$)	K'_m (mM)	K'_i (mM)	k'_{cat} (s^{-1})	k'_{cat}/K'_m ($M^{-1}s^{-1}$)
acetaldehyde	158	n.d. [b]	5.6	36	n.d.	n.d.	n.d.	n.d.
propanal	38.9	46.6	55	$1.4\ 10^3$	27	n.d.	84.0	306
butanal	2.76	16.6	57	$2.1\ 10^4$	23.1	n.d.	47.3	$2.1\ 10^3$
2-methylbutanal	1.85	2.74	113	$6.1\ 10^4$	17.7	4.61	92.3	$5.2\ 10^3$
3-methylbutanal	0.21	7.14	74.9	$3.6\ 10^5$	1.89	253	91.8	$4.9\ 10^4$
pentanal	0.16	3.90	56.6	$3.6\ 10^5$	3.01	13.9	81.6	$2.7\ 10^4$
hexanal	0.18	0.79	71.1	$4.0\ 10^5$	0.83	2.80	50.1	$6.1\ 10^4$
heptanal	0.27	0.88	72.9	$2.7\ 10^5$	4.25	1.17	101	$2.4\ 10^4$
hexanol	1.22	n.d.	2.55	$2.1\ 10^3$	- [c]	-	-	-
t-2-hexenol	1.69	n.d.	14.0	$8.3\ 10^3$	-	-	-	-

[a] Apparent values (K'_m, K'_i and k'_{cat}) were determined with 0.15 mM NADPH or 0.15 mM NADH.
[b] n.d., not detectable
[c] -, not performed

Effect of pH and ionic strength

Ionic strength had a significant influence on enzyme activity. Increasing the ionic strength of the standard assay (I = 0.03 M) by addition of NaCl, KCl or Na_2SO_4, increased the rate of reduction with NADPH, whereas the activity with NADH was severely inhibited. Concentrations of 200 mM KCl or NaCl increased activity with NADPH five to six-fold and decreased NADH activity with the same order of magnitude.

Reducing activity was tested at a wide pH-range. As ionic strength influences activity, conditions with high (I=0.1 M), as well as low (I=0.01 M) ionic strength were used (Fig. 3). At high ionic strength, NADPH-dependent activity was high over a broad pH range (6.5 to approx. 8.5), and was decreased over the entire pH interval at lower ionic strength; here an optimum pH of 8.5 was observed (Fig. 3A). The reverse effect was observed with NADH (Fig. 3B). Here, activity was highest at low ionic strength with an optimum between pH 6 to 7. Since the pK_a of the 2'-phosphate of NADPH is 6.1 in solution [4], deprotonation of this phosphate

group may facilitate binding, explaining the relatively high pH optimum observed for the reaction with NADPH.

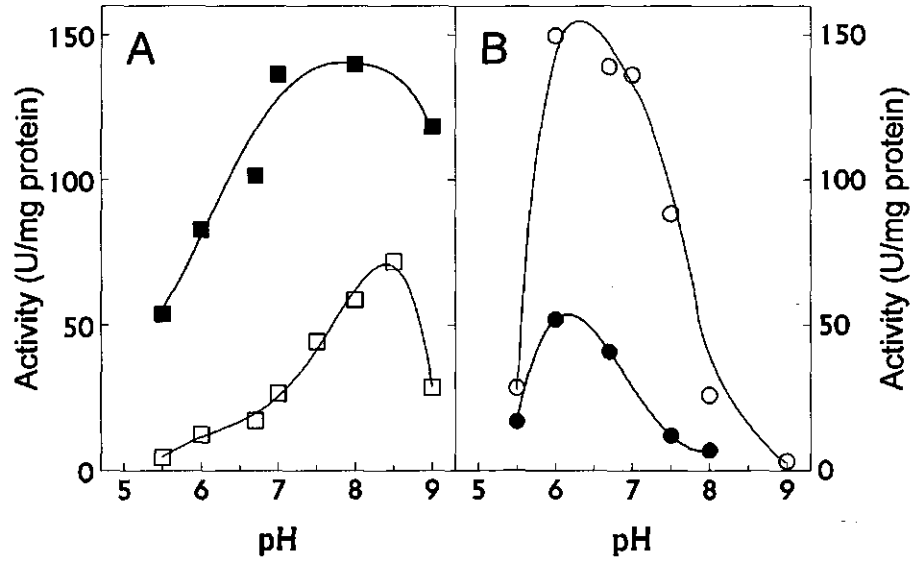

Figure 3. **Relative activity of purified ADH at various pH values. Reduction of 3-methylbutanal at low ionic strength, *I*=0.01 M (□, ○), and at high ionic strength, *I*=0.1 M (■, ●). Reduction with NADPH (A), and with NADH (B) are shown. Experiments were performed in 10 mM MES, MOPS and Tris. KCl was used to increase the ionic strength.**

Enzyme properties

The results show that the purified NADP-dependent ADH has a preference for long and branched-chain substrates with up to seven carbon atoms. The increase in catalytic efficiency with substrates of increasing chain length is opposite to results reported for ADH1-4 [3, 9, 10, 19, 20]. The bcADH is also different from the constitutively expressed ADH described by Wales and Fewson [18], since M_r (37 vs 46 kDa), coenzyme specificity (NADH/NAD^+ conversion was reported not

to occur), optimum pH, and the regulation of expression differ. In addition, we observed a remarkable influence of ionic strength on the enzyme activity, not reported previously. Substrate and coenzyme preferences, combined with its structural properties such as low pI and monomeric structure indicate that we purified a novel NADP-dependent bcADH.

Physiological function

During sugar fermentation in rich media, fusel alcohol concentrations generally reach values well above the flavour threshold value [11]. At 30°C, the estimated production rate of 3-methylbutanol during fermentation reaches values up to 0.6 µmol min^{-1} g^{-1} DW [12]. With a soluble protein concentration of about 200 mg g^{-1} DW (i.e. half of the total protein), the minimal specific activity for the NADP-dependent alcohol dehydrogenase must be at least 3 mU mg^{-1} protein. Since we measured 16 mU mg^{-1} protein, the bcADH could easily account for the entire production of 3-methylbutanol during fermentation.

The physiological role of the bcADH is not known. A possible function may be towards maintaining the $NADP^+$/NADPH balance. No transhydrogenases have been found in *S. cerevisiae* and the organism uses different strategies to maintain a proper redox balance [8]. Under aerobic conditions, NADH and NADPH can be oxidized by mitochondrial respiration. Under anaerobic conditions, the proper NAD^+/NADH balance is maintained by glycerol production at the expense of ATP. Overproduction of NADPH by the pentose phosphate pathway (PPP) can only be compensated for by NADPH-linked reductions [15]. The purified enzyme may therefore play an important role in the regeneration of $NADP^+$. Estimating the flux through the PPP during respirofermentation of glucose on a rich medium, Gancedo and Serrano [8] calculated a flux of 2.6 µmol hexose monomers g^{-1} min^{-1}, resulting in approx. 15 µmol NADPH g^{-1} min^{-1}. Thus, a small overproduction in the PPP already ensures sufficient NADPH for the reduction of branched-chain aldehydes.

Summarizing, a novel NADP-dependent branched-chain alcohol dehydrogenase, which is expressed under anaerobic conditions, was purified from *S. cerevisiae*. The enzyme may have a significant influence on flavour formation during alcoholic fermentations.

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INFLUENCE OF IMMOBILIZATION ON FERMENTATION AND ALDEHYDE REDUCTION DURING THE PRODUCTION OF ALCOHOL-FREE BEER

Abstract

Production of alcohol-free beer by limited fermentation is optimally performed in a packed-bed reactor. This highly controllable system combines short contact times between yeast and wort with the reduction of off-flavours to concentrations below threshold values. In the present study, the influence of immobilization of yeast to DEAE-cellulose on sugar fermentation and aldehyde reduction was monitored. Immobilized cells showed higher activities of hexokinase and pyruvate decarboxylase compared to cells grown in batch culture. In addition, a higher glucose flux was observed, with enhanced excretion of main fermentation products, indicating a reduction in the flux of sugar used for biomass production. ADH activity was higher in immobilized cells compared to that in suspended cells. However, during prolonged production a decrease was observed in NAD-specific ADH activity, whereas NADP-specific activity increased in the immobilized cells. The shifts in enzyme activities and glucose flux correlate with a higher *in vivo* reduction capacity of the immobilized cells.

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Introduction

Yeast immobilization is known to influence cellular metabolism [1, 2]. Cells of *Saccharomyces cerevisiae* entrapped in calcium alginate, were shown to have a higher glucose flux, more substrate was channelled to biomass and ethanol [3]. Intracellular pH was found to be decreased, whereas glucose phosphorylation was increased [4]. In addition, the amounts of storage and structural carbohydrates increased, as did the number of ploidy [5]. Due to the decrease in growth rate, amino acid metabolism decreases and increased concentrations of oxo-acids are released into the fermentation medium [3, 6].

Given the shifts in cell metabolism it has proven difficult to translate traditional brewing production processes into an immobilized and continuous process. However, in the production of novel beer types such as alcohol-free beer, these changes in cell metabolism can be exploited [7, 8]. Here yeast is immobilized to the surface of the carrier, a DEAE-cellulose based granular material. During the limited fermentation at low temperature and anaerobic conditions, cells reduce wort off-flavours and produce a beer flavour. The main wort off-flavours are aldehydes, such as 2- and 3-methylbutanal, hexanal, and heptanal, which are reduced to the corresponding alcohols. In addition, intracellular aldehydes, formed as intermediates in cell metabolism are reduced to fusel alcohols, e.g. 1-propanol, isobutanol, and isoamyl alcohol [8, 9].

Reduction of aldehydes in *S. cerevisiae* is performed by alcohol dehydrogenase (ADH) [10, 11]. Several ADHs are present in yeast, most of which are dependent on the coenzyme NAD. ADH1 is the main fermentative enzyme, and its functioning is essential under anaerobic conditions. ADH2 is glucose-repressed but when cells grow on ethanol, it is the main enzyme oxidizing ethanol to acetaldehyde. Other NAD-specific isoenzymes have been reported to exist but so far their significance in yeast metabolism and flavour formation has not been clarified [10, 11]. Recently, we purified an NADP-dependent alcohol dehydrogenase which proved to be highly specific for the reduction of branched-chain aldehydes. This enzyme may thus have an important function in the reduction of 2- and 3-methylbutanal during production of alcohol-free beer [12].

In the present article, we focused on the production of alcohol-free beer with immobilized cells of *S. cerevisiae*. Aldehyde reduction, enzyme activities, and the relation with sugar fermentation in immobilized cells was investigated.

Materials & Methods

Strain and growth conditions

A bottom-fermenting brewer's yeast strain of *Saccharomyces cerevisiae* var. *uvarum* strain W34, was provided by the Institute of Weihenstephan, Technical University of Munich, Germany. *S. cerevisiae* W34 was grown in wort (12°Plato). Aerobic batch growth was performed in a temperature-controlled rotary shaker operated at 150 rpm using shake flasks. Anaerobic growth was performed in wort, supplied with ergosterol and unsaturated fatty acids as described by Visser *et al.* [13]. Batch growth was performed at12°C, and followed by measuring the optical density (OD) increase at 600 nm.

Reactor operation

Production of alcohol-free beer was carried out by a limited fermentation using a packed-bed reactor, operated under down-flow conditions. The reactor with a volume of 1.5 m^3, was loaded with 1 m^3 of carrier. Flow rate and temperature were set at approximately 0.5 m^3 h^{-1} and 4°C. Dependent on yeast growth and product concentration, temperature and flow rate were adjusted to values of 0 to 2°C and 2 m^3 h^{-1}. Generally, the reactor was operated for 5 to 7 months.

In the reactor, the yeast cells were attached to the surface of the carrier. This carrier was a granular material consisting of polystyrene coated with DEAE-cellulose, and was provided by Cultor®Ltd., Helsinki, Finland. The material is non-porous and not-compressible and particle sizes range from 0.3 to 0.8 mm. The carrier is inert and can be sterilized at 80°C with NaOH (2% w/v). These characteristics enable its use in a packed-bed reactor.

Cell sampling and preparation of cell extracts

For determination of enzyme activities, carrier material was regularly taken from the reactor. Cells were suspended by washing the carrier 5 times in physiological salt solution and sonicating it each time for 2 min in a waterbath-sonicator. During the entire procedure, samples were kept on ice. In case of aerobic or anaerobic batch growth, cells were harvested at the end of the logarithmic growth phase. Cells were broken using glass beads as described by Van Iersel *et al.* [14], and cell extract (CE) was stored at -80°C when not used immediately.

Enzyme assays

Spectrophotometric assays were carried out with a Beckman DU-60 spectrophotometer, supplied with a Kinetics Soft-Pac module and temperature incubator set at 30°C (Beckman, Fullerton, USA). Absorption was measured at 340 nm, and reaction rates were linearly proportional to the amount of extract added. One unit is defined as the conversion of 1 µmol NAD(P)H per min.

Hexokinase (HEX): 50 mM imidazole HCl buffer, pH 7.6, 10 mM $MgCl_2$, 1 mM $NADP^+$, 50 U glucose-6P dehydrogenase, 10 mM D-glucose. The reaction was started with 1 mM ATP.

Glucose-6P dehydrogenase (G6PDH): 50 mM imidazole HCl, pH 7.6, 10 mM $MgCl_2$, 1mM $NADP^+$, 1 mM glucose-6P.

Pyruvate decarboxylase (PDC): 40 mM imidazole HCl, pH 6.5, 5 mM $MgCl_2$, 0.15 mM NADPH, 0.2 mM thiamine pyrophosphate, 70 U ml^{-1} alcohol dehydrogenase, 50 mM pyruvate.

NAD-dependent alcohol dehydrogenase (ADH): 50 mM glycine KOH, pH 9.5, 1 mM NAD^+, 100 mM ethanol.
*Branched-chain alcohol dehydrogenase (*bcADH*)*: buffer pH 6.7, containing 30 mM of each MES, MOPS and Tris, 0.15 mM NADPH, and enzyme. Reaction was started by addition of 3-methylbutanal to a final concentration of 10 mM, according to Van Iersel *et al.* [12].

IEF gel activity stain

ADH activity in gels was detected according to Seymour and Lazarus [15] with minor modifications. Instead of native gels, we used isoelectric focusing gels and a PhastSystem for separation (Pharmacia Biotech, Uppsala, Sweden). Gels were soaked in 20 mM BisTris, pH 7.0, containing 2 mM NADH or NADPH for 15 min on ice. Excess of buffer was drained and the gel was covered with filter paper strips soaked in substrate solution, i.e. 10 mM hexanal or 10 mM 3-methylbutanal in 20 mM BisTris, pH 7.0. After approx. 5 min, filter paper was removed and gels were exposed to ultraviolet light and analyzed for disappearance of fluorescence indicating reductase activity.

Fermentation assay

To determine the rate of product formation per biomass, carrier material was taken from the reactor and incubated anaerobically in wort of 12°P. Incubation occurred at two different temperatures, 2°C and 12°C. At regular time intervals, culture samples were taken, filtered over a 0.45 µm filter, and subsequently frozen. The concentrations of the different flavour components were determined by headspace analysis using gas chromatography (GC-HS).
To determine *in vivo* reducing capacity, similar experiments were performed in the presence of aldehydes. A stock solution of 3-methylbutanal, hexanal and heptanal in wort was prepared by sonication as described by Van Iersel *et al.* [12]. Aldehydes were added to the cell suspension to a final concentration of 0.3 mM and concentrations of flavour components were determined as described below.

Analytical methods

Flavour components were determined by static headspace analysis using gas chromatography (GC-HS). The gaschromatograph (HRGC 5300 Mega series, Carlo Erba Instruments, Milan, It.) was supplied with a cold trap. Samples were incubated for 30 min at 60°C in a temperature incubator (HS800, Fison Instruments, Interscience, Breda, The Netherlands) and were rotated for 30 sec at 1500 rpm with 30 sec time intervals. 2.5 ml of headspace was taken and injected in the cold trap (-110°C). After purging the cold-trap, sample was injected on the column (DB-Wax, 30 m x 0.542 mm, film 0.1 µm, J & W, Interscience) by heating the trap to 240°C (MFA 815, Fison Instr.) with helium as carrier gas (0.3 kPa). A temperature program was used starting at 30°C and heating the oven at 2.5°C min^{-1} to 110°C. Detection occurred with an FID-detector (EL 980, Fison Instr.).
Sugars, ethanol, glycerol and acetic acid were determined by HPLC (SpectraPhysics, San Jose, USA) using an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad, Veenendaal, The Netherlands) and a Shodex RIse-61 refraction index detector (Showa Denko, Tokyo). Elution was performed with 50 mM H_2SO_4 at 40°C and a flow rate of 0.6 ml min^{-1}. All eluents were prepared with distilled and Nanopure-filtered water.
Protein concentrations were determined according to the method of Lowry *et al.* [16]. Dry weights (DW) were measured by filtration over predried and preweighed nitro-cellulose filters (0.45 µm). Filters were dried at 80°C.

Results

Fermentation capacity

Sugar metabolism in immobilized and in suspended cells of *S. cerevisiae* during anaerobic fermentation in wort was monitored by analysis of substrate uptake and product formation, i.e. ethanol, glycerol and acetate (Table 1). During the time course of the experiment, a decrease was observed in the concentrations of sucrose, fructose, and glucose, whereas maltose remained constant (data not shown). It appeared that the total flux of sugar monomers was only slightly enhanced in the immobilized cells when compared to suspended, anaerobically grown cells. However, the immobilized cells showed a significant increase in the rate of formation of ethanol, glycerol and acetic acid (Table 1).

Table 1. **Fermentation by immobilized and suspended cells of *S. cerevisiae*. Immobilized cells were harvested during production of alcohol-free beer, suspended cells were harvested at the end of logarithmic growth in anaerobic batch culture.**

	Immobilized cells ($\mu mol\ h^{-1}\ g^{-1}$ DW) [a]	Suspended cells
sugar [b]	2420	2180
ethanol	4430	3670
glycerol	270	177
acetic acid	49	29

[a] Average activity measured during 75 days of operation.
[b] Summation of glucose, fructose, sucrose and maltose. Calculated in C6-monomers.

Enzyme assays with extracts of immobilized cells revealed that during the entire alcohol-free beer production period, the activities of hexokinase (HEX), glucose-6P dehydrogenase and pyruvate decarboxylase (PDC) were constant.

Table 2. **Enzyme activity in cell extract from *S. cerevisiae*. Exponentially growing cells were harvested during alcohol-free beer production, or during batch growth in wort (12°P) at 12°C under anaerobic conditions.**

Enzyme	Immobilized anaerobic cells [a] ($U\ mg^{-1}$ protein)	Suspended anaerobic cells
hexokinase	1.93 ± 0.07	1.03 ± 0.09
glucose 6P-dehydrogenase	0.092 ± 0.007	0.109 ± 0.002
pyruvate decarboxylase	2.12 ± 0.39	1.71 ± 0.15

[a] Average activity measured during 75 days of operation.

Compared to extracts of cells grown in suspended batch culture, HEX and PDC appeared to be increased, whereas the activity of G6PDH was substantially decreased in the immobilized cells (Table 2).

Fermentation was further analyzed by determining the production rate of fusel alcohols. The formation of the fusel alcohols 1-propanol and isobutanol was more or less constant during the entire alcohol-free beer production period. However, the production rate of isoamyl alcohol decreased slightly (Fig. 1).

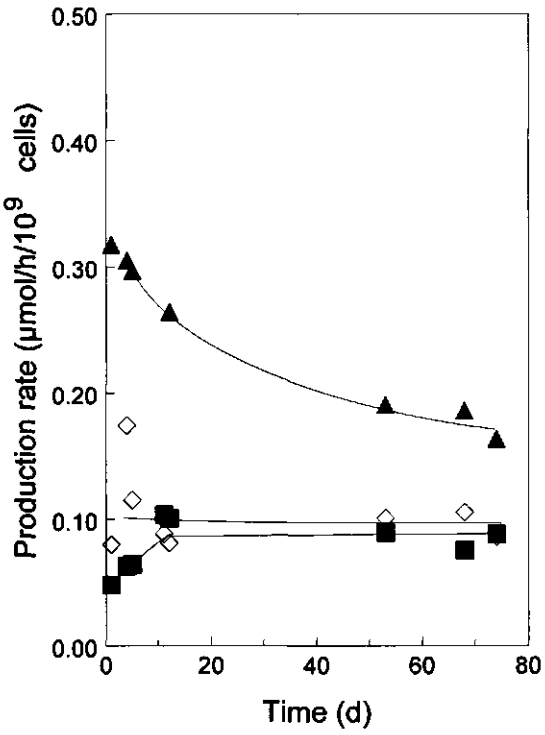

Figure 1. **Rate of fusel alcohol formation by *S. cerevisiae*. Cells were harvested during production of non-alcohol beer and incubated in wort. Flavour production rates were measured during anaerobic fermentation at 12°C, 1-propanol (◊), isobutanol (■) and isoamyl alcohol (▲).**

Aldehyde reduction

Saccharomyces cerevisiae var. *uvarum* is well able to reduce aldehydes. In the presence of glucose, hexanal, heptanal and octanal were almost

completely reduced within two hours. This was not observed in the absence of glucose, indicating that metabolic activity is necessary for aldehyde reduction (Fig. 2). Similar results were obtained with other linear aldehydes, such as pentanal, nonanal, decanal and undecanal, and with 2- and 3-methylbutanal (data not shown).

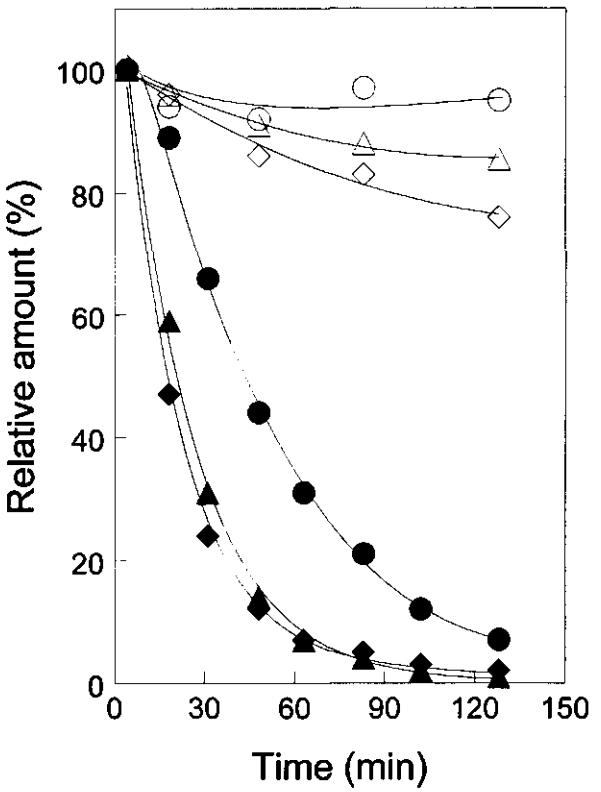

Figure 2. **Aldehyde reduction by cells of *S. cerevisiae*. Exponentially growing cells were suspended in a buffer containing 50 mM glucose (closed symbols), or without glucose (open symbols). Hexanal (●,○), heptanal (▲,△) and octanal (♦, ◊) were added each at concentrations of approx. 0.3 mM.**

The reducing capacity of the yeast cells was measured by incubation of cells with a mixture of 3-methylbutanal, hexanal and heptanal, each at a final concentration of 0.3 mM. Reduction appeared to be significantly higher in the immobilized cells compared with suspended cells. A reduction rate of 7.3 µmol min^{-1} g^{-1} DW was calculated for the immobilized cells, whereas with suspended cells the rate was 4.3 µmol min^{-1} g^{-1} DW. In addition, the formation of the corresponding alcohols was also the highest in immobilized cells; values of 4.7 and 2.2 µmol min^{-1}

g^{-1} DW were obtained for immobilized and suspended cells, respectively. With both immobilized and suspended cells, the ratio of alcohol production over aldehyde reduction was less than one. Generally, conversion of heptanal to heptanol was approximately 0.9, whereas with hexanal and 3-methylbutanal ratios of 0.6 and 0.5 were observed, respectively.

ADH activities

The activity of the NAD-specific alcohol dehydrogenases (ADHs) of *S. cerevisiae* was determined during production of alcohol-free beer (Fig. 3). The activity decreased substantially, especially during the first five days. ADH activity was also determined in batch grown cells under anaerobic conditions. Here, activity was approximately 0.2 U mg^{-1} protein, which was similar to the activity detected in immobilized cells at the end of the production period (Fig. 3).

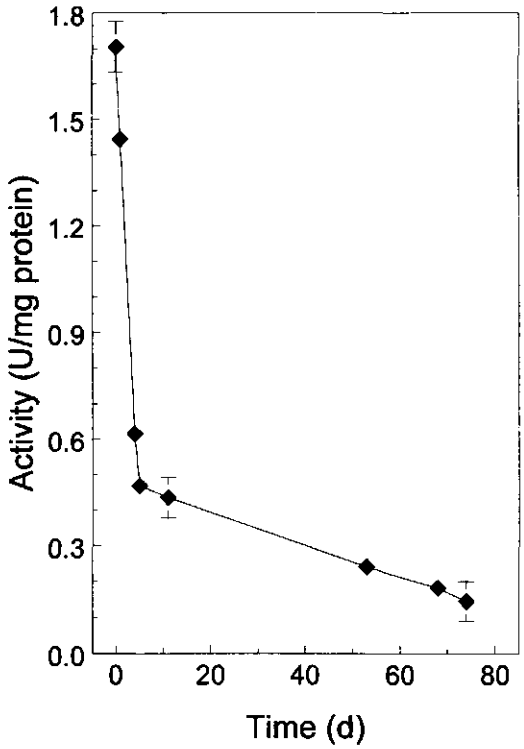

Figure 3. **Activity of NAD-specific alcohol dehydrogenases in cell extract from immobilized *S. cerevisiae*. Cells were harvested during production of alcohol-free beer, and activity was determined according to Materials and Methods.**

Recently, we purified and characterized an NADP-dependent branched chain alcohol dehydrogenase (bcADH) from *S. cerevisiae* [12]. To determine its contribution to the reduction of wort aldehydes, cell extracts were separated on IEF gels, and reducing activity was analyzed using an activity stain with the coenzymes NADH or NADPH (Fig. 4). With NADPH, reductase activity in the anaerobic CE was detected with hexanal and 3-methylbutanal, residing at a pI of 5.9. With NADH, a large activity spot was observed in the CE lane with hexanal, indicating that additional enzymes were able to reduce this compound. On the other hand, with 3-methylbutanal, reductase activity in the presence of NADH resided in a distinct spot at pI 5.9, similar to the activity with NADPH.

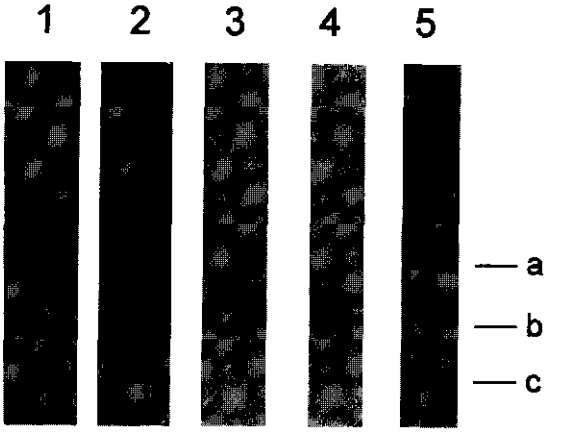

Figure 4. **IEF activity gels of cell extract from *S. cerevisiae* using the coenzymes NADPH or NADH as fluorescent stain. Lanes: 1; reduction of hexanal with NADPH, 2; reduction of 3-methylbutanal with NADPH, 3; reduction of hexanal with NADH, 4; reduction of 3-methylbutanal with NADH. Lane 5; purified bcADH, reduction of 3-methylbutanal with NADPH. [a] human carbonic anhydrase B, pI 6.55. [b] bovine carbonic anhydrase B, pI 5.85. [c] ß-lactoglobuline A, pI 5.2.**

In addition, *in vitro* NADP-reducing activity was determined in yeast cell extracts during the production of alcohol-free beer. In contrast to NAD-specific ADH, the NADP-specific activity increased from approx. 0.05 to 0.1 U mg^{-1} protein over a 70 day production period (Fig. 5). Activity in the immobilized cells was also significantly higher when compared to cells that were grown in aerobic or

anaerobic batch cultures, which had activities of 0.010 and 0.019 U mg^{-1} protein, respectively.

Discussion

Fermentation and reduction

According to Tables 1 and 2, rates of glycolysis were enhanced in immobilized cells compared to suspended cells. Both the activities of some major glycolytic enzymes and the conversion of substrate were higher. In addition, a relatively higher amount of sugar was converted to ethanol, glycerol and acetic acid in immobilized cells compared to suspended cells. Although micro-environmental conditions are expected to differ substantially between DEAE-cellulose immobilized cells and calcium-alginate entrapped cells, similar behaviour of the latter type has been reported in literature. In calcium-alginate entrapped yeast cells, both glucose uptake and ethanol production were increased [5]. In addition, Hilge-Rotmann and Rehm [17] observed an increase in hexokinase activity in gel-entrapped cells compared to freely suspended cells. They noticed also that this increase was less pronounced at very low cell densities in the gel, indicating that high cell density, a condition that is met with DEAE-cellulose immobilized cells, may be responsible for this increase in glucose flux, and decrease in cell yield [17]. In a packed bed reactor, lower cell yields are of significant importance since biomass formation shortens production periods due to pressure increase and blockage of flow.

The enzymatic reduction of aldehydes by *S. cerevisiae* is coupled to oxidation of the cofactors NADH or NADPH. Therefore, both NADH and NADPH have to be continuously regenerated. The major pathways which are involved in regeneration are glycolysis and the pentose phosphate pathway for NAD(H) and NADP(H), respectively [18]. Therefore, it may be expected that aldehyde reduction is correlated with the glucose flux. Indeed, aldehyde reduction was significantly decreased in the absence of glucose (Fig.2). In addition, immobilized cells, which appeared to have a higher glucose flux (Table 1), revealed a higher reduction capacity than suspended cells.

Role of ADHs

Several alcohol dehydrogenases are present in *S. cerevisiae* [10, 11]. Since stationary-phase, non-repressed cells are used to inoculate the alcohol-free beer reactor, both ADH1 and ADH2 are expected to be present. The significant decrease in ADH activity observed during the first five days of alcohol-free beer production can thus be explained by glucose-repression of ADH2 (Fig. 3).

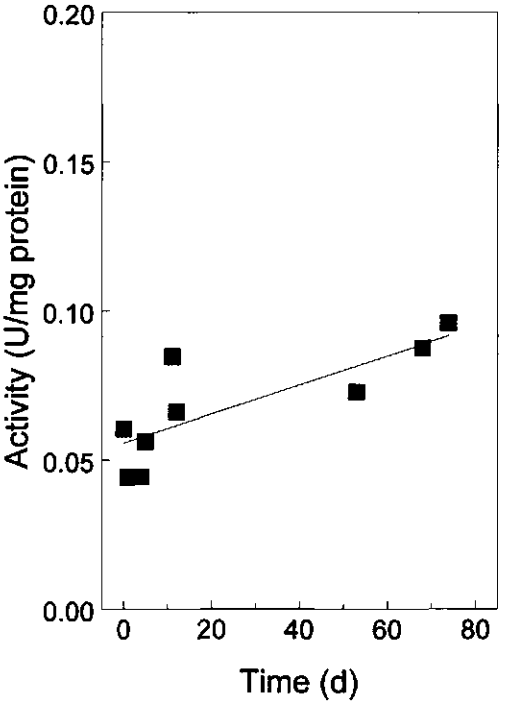

Figure 5. **NADP-specific alcohol dehydrogenase activity in cell extract from immobilized *S. cerevisiae*. Cells were harvested during alcohol-free beer production.**

Recently, we purified an NADP-dependent ADH with high specificity towards branched-chain aldehydes. At low ionic strength, this enzyme was capable of using NADH as a cofactor, whereas its activity wth NADPH was substantially lower [12]. This may explain the large activity spots in the IEF-gels, when tested with NADH (Fig. 4). However, under physiological conditions it is expected that this enzyme will use NADPH primarily [12]. According to Fig. 4, the bcADH appeared to be the only enzyme involved in the reduction of 3-methylbutanal. In addition, NADP-specific activity was observed to increase slightly during alcohol-free beer production (Fig. 5). This may in part be explained by the anaerobic conditions at which a high activity of the bcADH was observed [12]. Overall, NADP-specific activity was three to five-fold higher in immobilized cells compared to suspended anaerobic cells.

Metabolism of aldehydes

The main carbonyl compounds present in wort are formed by three different reactions, viz. Maillard reactions between amino acids and sugars, Strecker degradation of amino acids, and degradation of lipids [19]. Compounds formed in the latter two reactions are also intermediates of metabolic pathways of yeast and may thus be taken up by the cells and metabolized. In the experiments, it was observed that reduction of aldehyde on a molar base was higher than the production of the corresponding alcohol. This may be explained by metabolization of aldehydes as precursors for lipid synthesis. The ratio for heptanol/heptanal was close to one, suggesting that a carbon chain of 7 atoms is less favourably metabolized than 6 or 8 atoms. This is in line with the observation that the lipids of yeast cells are composed of fatty acids with an even number of C-atoms [20].

In conclusion, the results reported here show that cells immobilized to DEAE-cellulose have a higher rate of fermentation. A correlation was observed between this higher fermentation rate and an increased reduction capacity. In addition, immobilized cells appeared to have a higher *in vitro* ADH activity when tested for NADP-dependent activity. Thus, immobilization of yeast improves its reducing capacity during the production of alcohol-free beer.

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FLAVOUR FORMATION AND CELL PHYSIOLOGY DURING THE PRODUCTION OF ALCOHOL-FREE BEER WITH IMMOBILIZED *SACCHAROMYCES CEREVISIAE*

Abstract

Production of alcohol-free beer by limited fermentation is optimally performed in a packed-bed reactor operating in down-flow. This ensures a highly controllable system with optimal reactor design. In the present study, we report on changes in the physiology of immobilized yeast cells in the reactor. During production, a simultaneous increase in the activity of alcohol acetyl transferase, and formation of the esters ethyl acetate and isoamyl acetate were observed. In addition, the amount of unsaturated fatty acids decreased significantly. Since these phenomena coincided with a significant decrease in growth rate, we conclude that the anaerobic conditions, and the absence of substantial levels of unsaturated fatty acids in wort, limit cell growth during production and stimulate formation of acetate esters. Low temperatures (2°C) appeared to suppress production of α-acetolactate, whereas at 12°C substantial levels were obtained. An optimal and constant flavour profile of the alcohol-free beer can be achieved by introduction of regular aerobic periods to stimulate yeast growth. Temperature can be used to control the rate of growth as well as the rate of flavour formation.

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Introduction

Over the last 5 to 10 years, several methods have been developed for production of low-alcohol, or alcohol-free beer [1]. Both removal of alcohol, as well as limited fermentation are successfully applied. In the case of limited fermentation, production is most efficient when using an immobilized system, due to the advantages of immobilized systems to suspended batch systems, viz. high controllability, flexibility and high biomass concentration which allows short contact times [2, 3, 4]. Such a system was developed and is being applied successfully for several years already [5, 6]. During the production of alcohol-free beer, wort aldehydes are reduced by the activity of the alcohol dehydrogenases of the yeast [7], and optimally a beer flavour is formed without substantial levels of ethanol or off-flavours such as diacetyl [8].

Fermenting cells of *S. cerevisiae* generally produce high levels of estery flavours. Final levels in the end-product are dependent on environmental conditions as well as strain characteristics. Generally, factors that lead to more vigorous fermentations, such as high sugar concentrations, increased temperature and low levels of oxygen, will increase ester formation [9]. The acetate esters, such as ethyl acetate and isoamyl acetate, are the most pronounced group and are synthesized by alcoholysis of acetyl-coenzyme A. Since acetyl-CoA is an intermediate in lipid biosynthesis, ester production is closely linked to the metabolism of lipids. As a result, production is low during the growth phase when lipids are accumulated, and enhances significantly upon the increase of intracellular concentrations of acetyl-CoA during stationary phase [9]. Recently, Malcorps *et al.* [10] suggested that ester synthesis is related to the induction of alcohol acetyl transferase (AAT) rather than to increased availability of acetyl-CoA. This was corroborated by Fujii *et al.* [11], who showed that expression of the AAT gene is suppressed by oxygen and unsaturated fatty acids.

In fermentations performed by *S. cerevisiae*, α-acetolactate, an intermediate in amino acid metabolism, is synthesized from pyruvate by acetohydroxy acid synthase (AHAS) [12, 13]. Overproduction will lead to leakage from the cell into the medium, where it decarboxylizes into diacetyl, an important off-flavour in beer. Both, higher temperatures and increased oxygen concentrations were reported to increase the amount of α-acetolactate, by influencing the rate and extent of yeast growth [14]. In addition, a clear correlation exists between the maximum concentration of vicinal diketones and the minimum concentration of free amino nitrogen [15].

In the present article we report on the production of alcohol-free beer by a brewer's yeast strain of *S. cerevisiae*. In the reactor flavour formation and yeast physiology are changed due to the anaerobic conditions and immobilization.

Manipulating the flavour of the outflow from the reactor by controlling temperature and oxygen is discussed.

Materials & Methods

Strain and growth conditions

A bottom-fermenting brewer's yeast strain of *Saccharomyces cerevisiae* var. *uvarum* strain W34, was provided by the Institute of Weihenstephan (Technical University of Munich, Germany). *S. cerevisiae* W34 was grown in wort (12°Plato). Aerobic batch growth was performed in a temperature-controlled rotary shaker operated at 150 rpm using shake flasks. Anaerobic growth was performed in the same wort, ergosterol and unsaturated fatty acids were supplied as mentioned by Visser *et al.* [16]. Growth was followed by measuring the optical density (OD) increase at 600 nm.

Limited fermentation

Production of alcohol-free beer was carried out by a limited fermentation using a packed-bed reactor, operated under down-flow conditions [5, 6, 8]. The reactor volume of 1.5 m^3 is loaded with 1 m^3 of carrier. Flow rate and temperature are set at approximately 0.5 m^3 h^{-1} and 4°C. Dependent on yeast growth and product concentration, temperature and flow rate are adjusted to values of 0 to 2°C and 2 m^3 h^{-1}. Generally, the reactor is operated for 5 to 7 months.

In the reactor, the yeast cells are attached to the surface of the carrier. This carrier is a granular material consisting of polystyrene coated with DEAE-cellulose, and is provided by Cultor®Ltd. Finnsugar Bioproducts, Helsinki, Finland. The material is non-porous and not-compressible and particle sizes range from 0.3 to 0.8 mm. The carrier is inert and can be sterilized at 80°C with NaOH (2% w/v). The characteristics enable its use in a packed-bed reactor.

Cell sampling and preparation of cell extracts

For determination of enzyme activities and cell composition, carrier material was regularly taken from the reactor. Cells were detached by washing the carrier in a physiological salt solution and sonifying it mildly in a waterbath-sonicator. During the entire procedure, samples were kept on ice. In case of aerobic or anaerobic batch growth, cells were harvested at the end of the logarithmic growth phase. Cells were washed with 0.1 M BisTris buffer, pH 7.0, 1 mM DTT and stored in the same buffer at -80°C.

Cells were broken by vortexing with an equal volume of glass beads (diameter varying from 0.1 to 1 mm). Samples were vortexed (max. speed) five times for 45 s periods, alternating with 45 s cooling periods (on ice). Cell debris was analyzed microscopically, and generally more than 90% of the cells were broken. Cell debris was removed by centrifugation (45,000 x g, 20 min, twice), and the cell extract (CE) was stored in different aliquots at -80°C. The activities of the enzymes did not decrease significantly during the storage period.

Enzyme assays

During the *in vitro* enzyme assays, all activities were linearly proportional with the amount of protein and with time. Reaction conditions for enzyme assays were as follows:

Acetohydroxy acid synthase (AHAS): 100 mM KP_i, pH 8.0, 5 mM $MgCl_2$, 1 mM TPP, 100 mM pyruvate and 0.2 mM FAD. Reaction was stopped by addition of 3.5 µl 3 M H_2SO_4 per 200 µl reaction mixture. The α-acetolactate formed in the reaction is readily decarboxylated to acetoin or diacetyl upon heating in acid [17]. Both were determined by gas chromatographic analysis of the static headspace (GC-HS).
Alcohol acetyl transferase (AAT): The assay to determine the activity of alcohol acetyl transferase (AAT) was adopted from Malcorps and Dufour [18]. 50 mM KP_i pH 7.5, 15 mM isoamyl alcohol, 0.8 mM acetyl-CoA. Reaction was stopped by lowering the pH to approximately 3 by addition of 2 µl 3 M H_2SO_4 per 200 µl reaction mixture. The amount of isoamyl acetate was determined by GC-HS. One unit is defined as the activity which produces 1 µmol isoamyl acetate per min.

Rate assays

The rates of formation of esters and α-acetolactate were determined per unit biomass. Carrier material was taken from the reactor and was incubated anaerobically in wort of 12°P at two different temperatures, 2°C and 12°C. At regular time intervals, samples were taken, filtered over a 0.45 µm filter, and subsequently frozen. No influence of storage procedure on the flavour composition of the samples was observed. Esters and α-acetolactate were analyzed by gas chromatography as described in the following.

Analytical methods

Concentration of the flavour compounds was determined by gas chromatographic analysis of the static headspace (GC-HS). The gas chromatograph (HRGC 5300 Mega series, Carlo Erba Instruments, Milan, It.) was supplied with a cold trap. Samples were incubated for 30 min at 60°C in a temperature incubator (HS800, Fison Instruments, Interscience, Breda, The Netherlands) and were rotated for periods of 30 sec at 1500 rpm with 30 sec time intervals. During this incubation, α-acetolactate is readily converted to diacetyl [19]. 2.5 ml of headspace was taken and injected in the cold trap (-110°C). After purging the cold-trap, sample was injected on the column by heating to 240°C (MFA 815, Fison Instruments). Carrier gas was helium (0.3 kPa), the column was DB-Wax, 30m x 0.542 mm, film 0.1 µm (J & W, Interscience) and a temperature program was used starting at 30°C and heating the oven at 2.5°C min^{-1} to 110°C. Detection occurred with an FID-detector (EL 980, Fison Instruments). The detection limit of diacetyl with this system is estimated at 0.2 µM.
Protein concentrations were determined according to the method of Lowry *et al.* [20].
Dry weights (DW) were measured by filtration over predried and preweighed nitro-cellulose filters (0.45 µm). Filters were dried at 80°C.

Analysis of fatty acids and sterols

Cells were harvested, washed in 0.1 M MES buffer, pH 6.0 and spun down. Lipids were separated by three subsequent extractions with methanol/chloroform (ratio 1:2). The chloroform fractions were pooled and dried with a stream of N_2-gas. Fatty acid composition in the lipid fraction was determined by gas chromatography of the methyl esters following the methylization of the lipid fraction with anhydrous, acid methanol. Experimental details are according to Verheul *et al.* [21].
Sterol composition was determined by direct injection of the lipid fraction onto a capillary, apolar GC column SGE BP-1, 12 m x 0.53 mm, film 0.1 µm (SGE, Milton Keynes, UK).

Sterols were separated with a temperature gradient of 220 to 300°C using helium as carrier gas (0.21 kPa).

Results & Discussion

Fermentation and growth

Production of alcohol-free beer is performed at low temperature and under anaerobic conditions [6]. These conditions significantly influence cell composition and metabolism. Therefore, the fatty acid composition of immobilized cells, harvested during alcohol-free beer production, was determined. The composition changed significantly, both the average length and the amount of unsaturated fatty acids decreased substantially (Fig. 1). In addition, a small increase was observed in the total amount of fatty acids (data not shown).

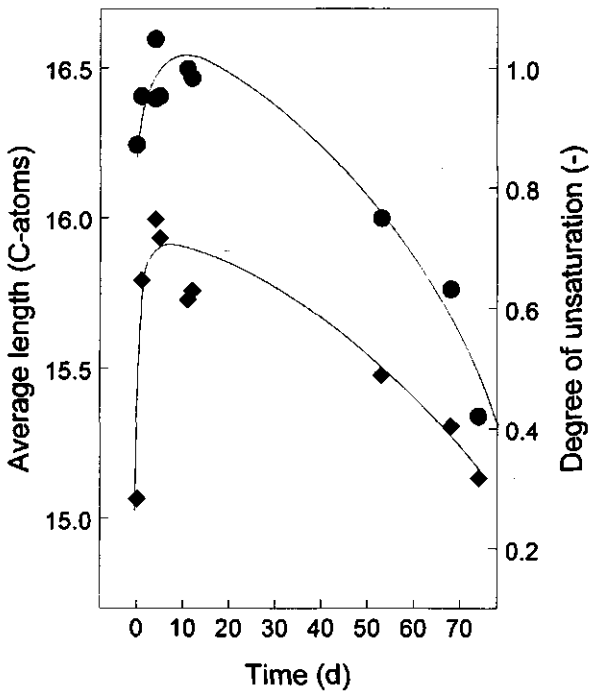

Figure 1. **Average chain length (●) and degree of unsaturation (♦) of fatty acids from *S. cerevisiae*. Cells were harvested during production of alcohol-free beer.**

In addition to fatty acid analysis, the sterol composition was determined during production. A significant increase was observed in the relative concentration of squalene after a prolonged production period, whereas concentrations of ergosterol and lanosterol decreased (Table 1). *S. cerevisiae* needs molecular oxygen to synthesize unsaturated fatty acids and sterols. In the synthesis of sterols, squalene is the last intermediate prior to the reactions which involve oxygen [22]. With suspended batch-grown cells, it has been shown that squalene accumulates during anaerobic conditions [23]. Apparently, this is also the case with immobilized *S. cerevisiae*.

Table 1. **Relative concentrations of squalene, ergosterol and lanosterol in *S. cerevisiae* during the production of alcohol-free beer.**

Time (d)	Squalene (%)	Ergosterol (%)	Lanosterol (%)
0	77	14	8
5	57	33	10
53	86	11	3
68	89	9	2
74	88	11	2

In theory the decrease in unsaturated fatty acids, as seen with cells growing in the anaerobic bioreactor, would result in a decrease of the fluidity of the membrane, whereas the simultaneous decrease in chain length has an opposite effect. The changes in degree of unsaturation and chain length apparently enable the cells to grow and maintain membrane function for several division cycles. When growth was determined in the bioreactor, cells indeed appeared to be able to grow for a period of approximately 20 days [24]. Although cells were continuously supplied with substrates and nutrients present in wort, growth decreased after this period since the wort only contained very limited amounts of unsaturated fatty acids (Van Dieren, pers. comm.).

The decrease in growth and the changes in membrane composition coincided with an increase in the formation of acetyl esters (Fig. 2). Both production of ethyl acetate and the production of isoamyl acetate were low during the first 20 days, but increased significantly following the decrease in growth rate. At higher fermentation temperature, an additional increase was observed, production rates at 12°C were approximately two to four fold higher compared to rates at 2°C (Fig. 2).

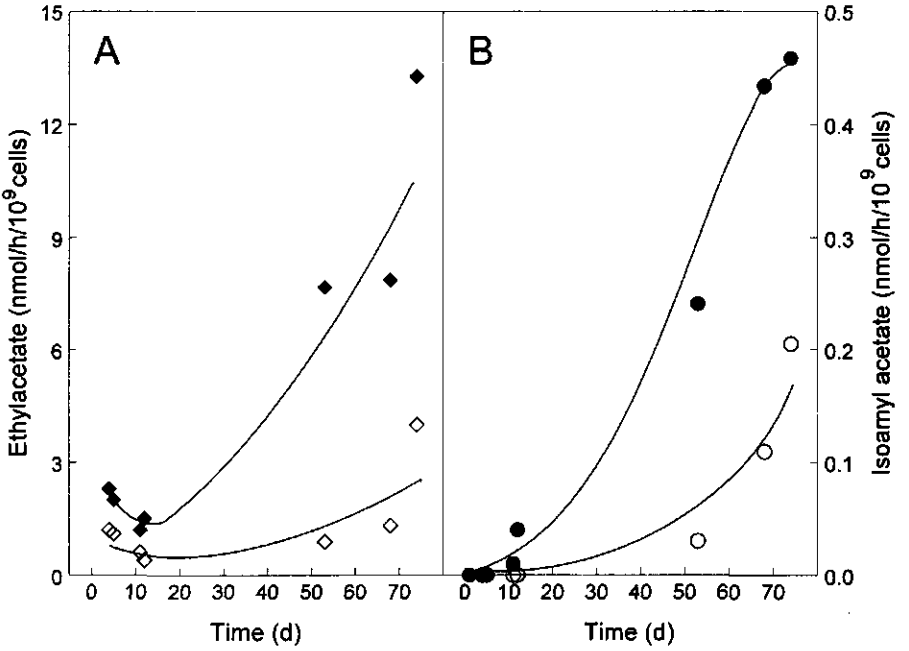

Figure 2. **Rate of ester formation by immobilized *S. cerevisiae*. Cells were harvested during production of alcohol-free beer and subsequently incubated in wort at 12°C (closed) or 2°C (open symbols) under anaerobic conditions. A. Formation of ethylacetate (♦, ◊). B. Formation of isoamyl acetate (●,O).**

Ideally, flavour formation is already high from the start of the production period. Therefore, we determined the activity of the ester producing enzymes, i.e. the alcohol acetyltransferases (AAT). It appeared that upon the decrease in the amount of unsaturated fatty acids, an increase in the activity of AAT was observed (Fig. 3). In addition, we determined AAT activity in batch grown cells (12°C). Under aerobic conditions, activity was approx. 0.005 $\pm$ 0.001 U g^{-1} protein, but was increased significantly under anaerobic conditions to 0.053 $\pm$ 0.007 U g^{-1} protein. Activity appeared to be comparable to the activity observed during prolonged alcohol-free beer production. The increase in AAT under anaerobic conditions coincides well with the low amount of unsaturated fatty acids, since enzyme synthesis is induced at low concentrations of these fatty acids [18, 11]. Therefore, we may conclude that during alcohol-free beer production, ester formation increases as a result of a decrease in unsaturated fatty acids.

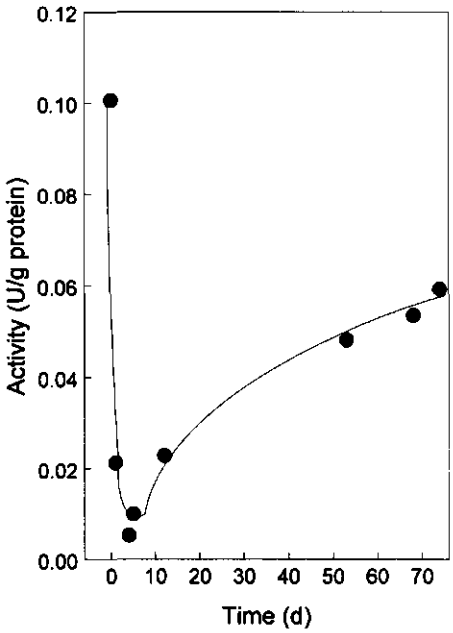

Figure 3. **Alcohol acetyl transferase (AAT) in cell extract from *S. cerevisiae*. Cells were harvested during alcohol-free beer production and AAT was measured according to Materials and Methods.**

Acetohydroxy acid synthesis

The flavour of beer is strongly affected by the amount of α-acetolactate, since during storage this is converted to the strong off-flavour diacetyl. Therefore, the *in vitro* activity of acetohydroxy acid synthase (AHAS) was determined. It appeared that enzyme activity was significant (38 ± 5 U g^{-1} protein), but comparable to values determined with batch grown cells (29 ± 3 and 25 ± 4 U g^{-1} protein for aerobically and anaerobically grown cells, respectively).

In addition, production of α-acetolactate was studied *in vivo* by fermentation of wort with cells harvested from the reactor (Fig. 4). Surprisingly, a clear influence of incubation temperature was observed. At 12°C, high levels of α-acetolactate were reached within a short time period. However, in the fermentations at 2°C no increase in α-acetolactate was observed, concentrations remained below the detection limit of the FID detector (0.2 µM). This confirmed results that were obtained during production of alcohol-free beer where at the low production temperatures no α-acetolactate was observed (data not shown).

Since α-acetolactate is entirely produced by anabolic processes, influence of temperature on synthesis can be explained by changes in metabolism. Apparently, the yeast cell is better able to balance the production of this compound to the demand for the production of isoleucine, leucine and valine at low temperatures, compared to higher temperatures [25].

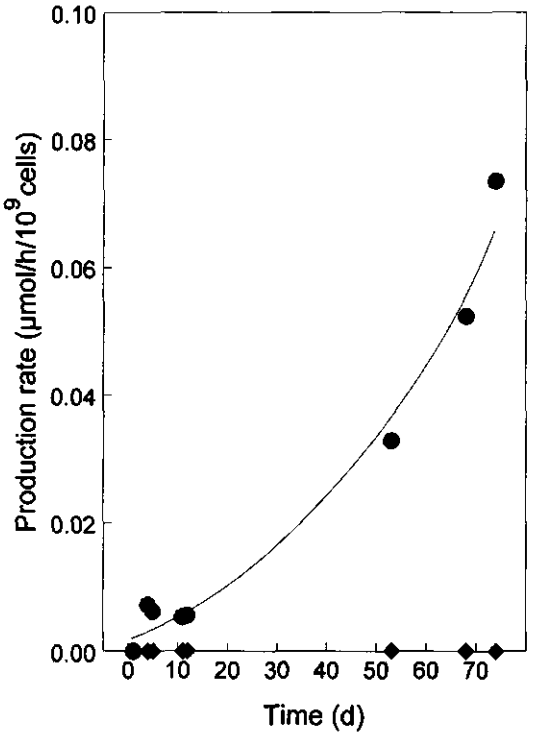

Figure 4. **Production rate of α-acetolactate by *S. cerevisiae*. Cells were harvested during production of alcohol-free beer, and subsequently incubated in wort of 12°P at 12°C (●) or 2°C (♦) under anaerobic conditions.**

Process control

During the production of alcohol-free beer, yeast metabolism is continuously affected by environmental conditions and wort composition. This enables the brewer to interfere and optimise the flavour profile of the final product. The anaerobic conditions inhibit growth and stimulate ester production, whereas oxygen stimulates growth but may cause oxidative off-flavours. By increasing temperature, yeast metabolism and ester production will increase. However, to

suppress acetohydroxy acid formation, temperature must remain sufficiently low, preferably between 0 and 4°C.

By introduction of regular aerobic intervals, an optimum can be reached between the supply of oxygen for yeast growth, and the prevention of oxidation of the alcohol-free beer. Intermittently, yeast metabolism is stimulated and cells are able to multiply. By varying the time of interval between two successive periods, cell physiology can be manipulated such that ester formation is constant during the entire production period. The continuous need for oxygen by the yeast will accomplish the complete removal of oxygen from the wort during alcohol-free beer production.

In addition, flow rate and wort composition are used to control flavour concentration. Early in production, biomass concentration is low, and thus flow rate is reduced. Depending on the increase in biomass, and subsequently the degree of fermentation (specific gravity) and production of flavours, the flow rate will be increased. For successful, reproducible fermentations leading to consistent beer quality, it is imperative that production is carefully monitored and controlled. The parameters mentioned above, enable a constant and optimal flavour profile of the alcohol-free beer.

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EFFECT OF ENVIRONMENTAL CONDITIONS ON FLOCCULATION AND IMMOBILIZATION OF BREWER'S YEAST DURING PRODUCTION OF ALCOHOL-FREE BEER

Abstract

Recently, a method using immobilized yeasts was developed and successfully applied for production of alcohol-free beer. In the present study, we investigated the influence of environmental conditions present during alcohol-free beer production on the flocculation and immobilization of the yeast *Saccharomyces cerevisiae* var. *uvarum*. In wort, the cells developed flocculation at the end of exponential growth, according to the NewFlo phenotype. In defined medium, the flocculation capacity appeared to be temporary, and was rapidly lost during the stationary phase. No increase in cell wall hydrophobicity at the onset of flocculation was observed in either medium. Low growth temperatures increased flocculation capacity approximately four-fold, compared to growth at high temperatures. However, both with cells grown at high and at low temperature, the optimum temperature for flocculation was at 25°C.

A novel method using carboxyfluorescein-stained cells was developed to analyse the initial adhesion of cells to carrier. This method also allowed rapid analysis of the effects of immobilization to DEAE-cellulose carrier during the alcohol-free beer production process. It appeared that a high flocculation capacity stimulated adhesion to the DEAE-carrier.

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Introduction

Production of low and alcohol-free beer is possible by different methods, which are often based on limited fermentation. Additional reduction in the alcohol content is obtained by different techniques such as dialysis, evaporation, and reversed osmosis [1, 12]. A limited fermentation can be achieved by combining high temperatures, 15°C to 20°C, with short contact times, 0.5 up to 8 hours, or by an extended contact time (up to 24 hours) at low temperature, 0°C to 5°C. Generally, high yeast cell concentrations are used (> 10^8 cells/ml) and thus fermentation must be carefully controlled in order to prevent an excess in alcohol production. In immobilized systems, the volume of yeast biomass can increase up to 15% of total volume compared to about 2% in batch systems, allowing a ten-fold reduction in fermentation time [11]. In the immobilized system that was developed for alcohol-free beer production [9, 25], granular particles are used as carrier for the yeast cells. This Cultor® carrier, a DEAE-cellulose based material, is used in a packed-bed reactor, which is operated under down-flow. The immobilization of yeast cells to the carrier is supposed to be based on charge differences between the negative cell walls and the positive DEAE-cellulose. However, positive charges of the DEAE-cellulose will be strongly reduced due to the adsorption of wort components (e.g. proteins and salts). Other parameters may thus be involved in the adhesion of yeast cells to the carrier surface. During immobilization, we noticed a high flocculation capacity of the yeast cells, a capacity that was retained during prolonged production.

Flocculation of yeast is defined as the phenomenon that cells aggregate in clumps and sediment or flotate rapidly from the medium. Specific interactions (molecular recognition), as well as non-specific interactions (double-layer, salt bridges, hydrophobic interaction) are involved in the flocculation, but the exact mechanism is still not fully understood, despite cloning of several flocculation (*FLO*) genes [16, 21]. Flocculation capacity is influenced by environmental conditions such as temperature, oxygen, and nutrient availability, and by strain characteristics. Flocculation of bottom fermenting yeast strains is initiated by Ca^{2+}, whereas sugars, such as mannose or glucose inhibit the flocculation. With ale yeasts, ethanol is the main inducer of flocculation [4, 5]. The involvement of sugar-specific inhibition indicated that a lectin-type protein is involved in flocculation, calcium may be needed to modify its conformation into an active receptor-binding conformation [10].

In the traditional brewing process, flocculation is an important parameter during fermentation. Its precise onset as well as the percentage of flocculating cells are important, and are both determined by environmental conditions and strain characteristics. Two parameters may be used to describe the

efficiency of the flocculation process; i) the rate of flocculation and ii) the relative amount of non-flocculated, single cells [18, 24]. The rate of flocculation is the rate at which cells adhere to a floc and can be measured by settlement of the flocs. The rate of flocculation is proportional with the incidence that two cells collide to each other and with the success of such a collision. Thus, an increase in agitation energy, as well as stronger bonds between yeast cells will determine the flocculation rate. At constant agitation energy and cell concentration, floc size and flocculation rate are therefore directly proportional with bond strength.

The second parameter is the ratio of suspended cells to flocculated cells. Flocculation is dependent on the strength of the cell-cell bonds within a floc, an increase in bond strength will increase the amount of cells that flocculate. In the case of a constant amount of total cells, the ratio is proportional with the free cell concentration, and may thus be used as a measure for bond strength.

The aim of the present study was to analyse in more detail the influence of environmental conditions on flocculation and immobilization of *Saccharomyces cerevisiae* var. *uvarum* W34. It appeared that both the growth temperature and the assay temperature had a pronounced effect on flocculation. Best temperature of flocculation assay was observed to be 25°C, whereas flocculation was significantly increased in cells grown at lower temperatures. No increase in cell wall hydrophobicity was observed at the onset of flocculation, indicating that this effect is strain-dependent and not a general characteristic of lager-yeast strains [4, 5, 7, 13]. A novel method using carboxyfluorescein-staining of cells was developed to analyse immobilization of yeast cells to DEAE-cellulose particles. Adhesion of the cells to DEAE-carrier appeared to be reduced significantly in rich-complex media, whereas under these conditions development of flocculation capacity resulted in a significant increase in the adhesion of yeast cells to carrier particles.

Materials and Methods

Strain and growth conditions

A bottom-fermenting brewer's yeast strain of *Saccharomyces cerevisiae* var. *uvarum* strain W34, was provided by the Institute of Weihenstephan, Technical University of Munich, Germany. *S. cerevisiae* W34 was grown in MYGP containing malt extract (3 g/l), yeast extract (3 g/l), glucose (10 g/l) and mycological peptone (5 g/l). Additionally, cells were grown in wort (12°Plato) or in a mineral medium with ammonium as the sole nitrogen, and glucose or maltose as the carbon and energy source [3]. Incubations were performed in a temperature-controlled rotary shaker operated at 150 rpm. Growth was followed by measuring the optical density (OD) increase at 600 nm.

Production of non-alcohol beer

Production of non-alcohol beer was carried out as described previously [9, 22, 25]. A limited fermentation was performed using a packed-bed reactor, operated under down-flow conditions. The volume of the reactor was 15 hl and was loaded with 10 hl of carrier. The carrier is a granular material consisting of polystyrene coated with DEAE-cellulose, and was provided by Cultor®Ltd. Finnsugar Bioproducts, Helsinki, Finland. The material is non-porous and not-compressible and particle sizes range from 0.3 to 0.8 mm. The carrier is inert and can be sterilized at 80°C with NaOH (2% w/v). Before production was started, a 24 h procedure was used to inoculate the reactor and immobilise the cells to the carrier. During production, flow rate and temperature were set at approximately 5 hl/h and 4°C. Dependent on the overall performance of the reactor, temperature and flow rate were adjusted to values of 0 to 2°C and 20 hl/h. Generally, the reactor was operated for 5 to 7 months.

Flocculation assay

Flocculation was measured according to Smit *et al.* [13]. During growth, cells were harvested (1,000 g, 5 min) and washed twice with phosphate buffer (50 mM, pH 6.0). Flocculation capacity was determined in a sodium/acetate-buffer (50 mM, pH 4.5) with 7 mM $CaCl_2$ or 2.5 mM Na-EDTA (control). The final amount of yeast biomass in the assay was 15 g DW l^{-1}. After 30 min incubation at 25°C (unless stated otherwise), the suspension was whirlmixed for 20 sec, 5 times inverted, and the optical density at 620 nm was measured for 3 min. All measurements were performed in duplicate or triplicate.
The data of the OD decrease during the flocculation assay were fitted to a sigmoid function. Flocculation rate was defined as the maximum decrease in absorbance per minute, i.e. the rate of decrease in the point of inflection of the curve.

Contact angle measurements

To determine the hydrophobicity of the yeast cell wall, contact angles of water with yeast cell walls were measured according to Smit *et al.* [13]. Cells were collected, washed twice and resuspended in a small volume of KP_i buffer (50 mM, pH 6.0). Cells were collected onto a 0.2 µm filter (Schleicher and Shell), and the filter was washed with an equal volume of KP_i buffer and dried at room temperature. To prevent formation of cracks in the cell layer, the filter was mounted to a glass slide and stored in a petridish, previously moisturized with a few drops of water. After drying, contact angles were measured using a microscope equipped with a goniometric eyepiece (Krüss GmbH, Hamburg). Each reported contact angle is the average of 10 to 15 independent measurements.

Adhesion

Adhesion of cells to carrier particles was measured by the decrease in OD (600 nm) caused by the addition of carrier to a yeast suspension. Prior to the assay the carrier was hydrated with hot tap water (approx. 65°C), and washed with demineralized water or with medium (MYGP or wort). 10% (w/v) of carrier was added to a suspension of yeast cells with an OD of 0.6, and incubated for 15 min with continuous shaking on a rotary shaker (150 rpm). By measuring the final OD of the suspension, after settlement of carrier particles, the number of cells which adhered to the carrier could be calculated. As a control, the experiment was performed without addition of carrier. All experiments were performed in triplicate and varied less than 20%.

Fluorescent labelling

To determine the adhesion of cells to carrier, a novel method was developed based on fluorescent labelling. Cells were labelled using carboxyfluorescein diacetate (cFDA), a non-fluorescent marker which becomes fluorescent upon hydrolysis into carboxyfluorescein (cF) by intracellular esterases. Due to the relatively high intracellular pH_2 cF is negatively charged and is retained within the cells which become brightly fluorescent [2]. Carrier was taken from the reactor and cFDA (10 mg ml^{-1} in acetone) was added to a final concentration of approx. 50 µM. Subsequently, cells were incubated for 5 min at 25°C and analyzed using a fluorescence microscope equipped with a camera (Zeiss Axioskop, Oberkochen, Germany).

Results

Flocculation characteristics

Flocculation was induced by addition of Ca^{2+}. Other cations like Mg^{2+}, Mn^{2+} or Cu^{2+} also induced flocculation although higher concentrations were needed. Mannose, added prior to whirlmixing, was able to inhibit flocculation at very low concentrations. pH optimum for flocculation is 4.5 (data not shown). Irreversible inhibition of flocculation occurred by incubation of cells with proteinase K and pronase E (data not shown). The flocculation thus corresponds with the mechanism proposed by Miki *et al.* [10], in which flocculation is mediated by lectins that bind mannose residues and require calcium to gain the active conformation.

Temperature of growth

Cells were grown at 4°C and 25°C to investigate the influence of temperature on flocculation. At various stages of growth, cells were harvested and the flocculation capacity was measured (Fig. 1). Cells did not flocculate when growing exponentially on glucose. With cells grown at high temperature, flocculation started at an OD of 5 (Fig. 1A), whereas cells grown at low temperature already started flocculating at an OD of approx. 1 (Fig. 1B). In addition, flocculation of cells grown at low temperature appeared to be much higher, compared to cells grown at 25°C; rates of 8 respectively 2.5 ΔA_{620}/min were measured.

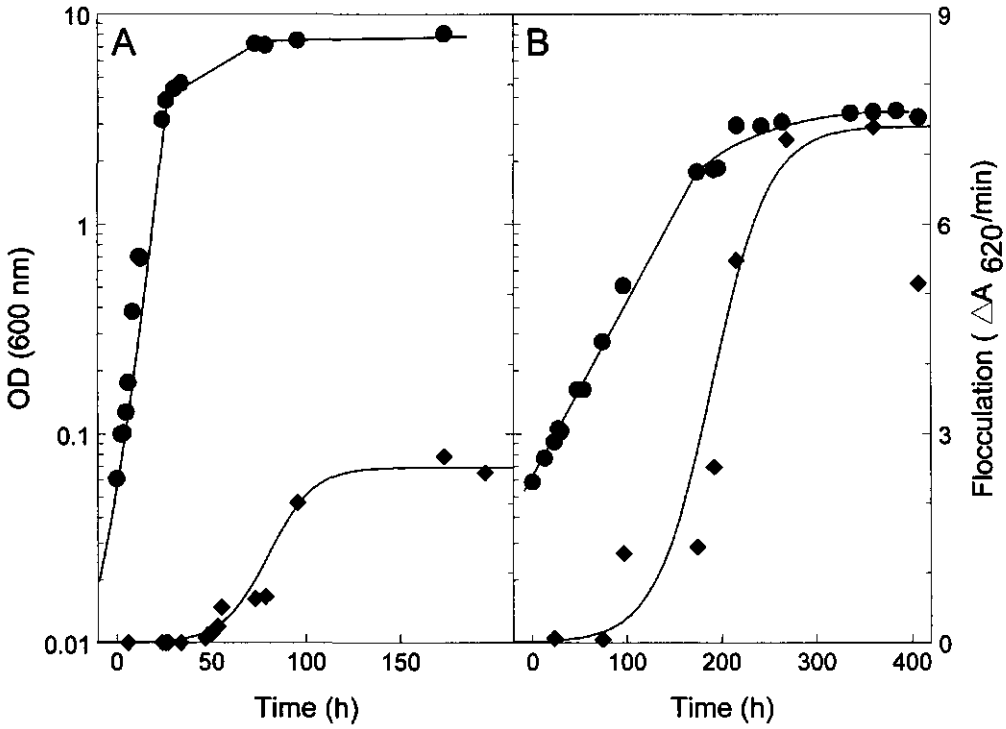

Figure 1. **Flocculation capacity at 25°C of cells of *S. cerevisiae* grown aerobically on MYGP at 25°C (A) and 4°C (B). ● OD (600 nm). ◆ Flocculation capacity (ΔA_{620}/min).**

Effect of medium composition

Flocculation capacity was further investigated by culturing cells in three different media: MYGP, wort of 12°P and a mineral medium. In general, no differences were found between flocculation of cells grown on wort or MYGP. With the mineral medium, however, flocculation capacity started immediately after exhaustion of the sugar, at the onset of oxidative growth (Fig. 2), and ceased at prolonged growth into stationary phase. When maltose was used as the carbon source instead of glucose, similar flocculating behaviour was observed (data not shown).

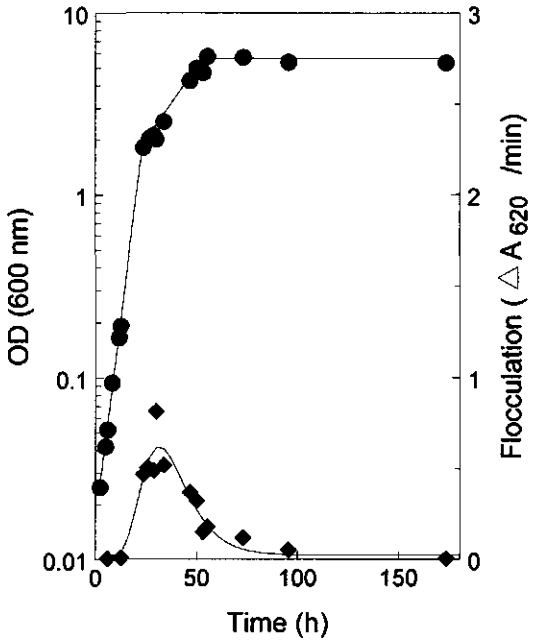

Figure 2. **Flocculation capacity of cells grown aerobically on mineral medium (25°C). ● OD (600 nm). ◆ Flocculation capacity (ΔA620/min).**

Temperature of flocculation assay

To investigate the effect of temperature on the flocculation process, the assay was performed at different temperatures with cells grown at 25°C and 4°C. Flocculation rate increased over the temperature range of 0 to 25°C, whereafter it decreased again at higher temperatures (Fig. 3A). In addition, the amount of non-flocculating, single cells was also temperature dependent. At 25°C, most cells flocculated, at higher as well as at lower temperatures an increasing amount of cells remained in suspension (Fig. 3B). Compared to cells grown at 4°C, cells grown at 25°C showed relatively poor flocculation behaviour over the entire range of flocculation temperatures.

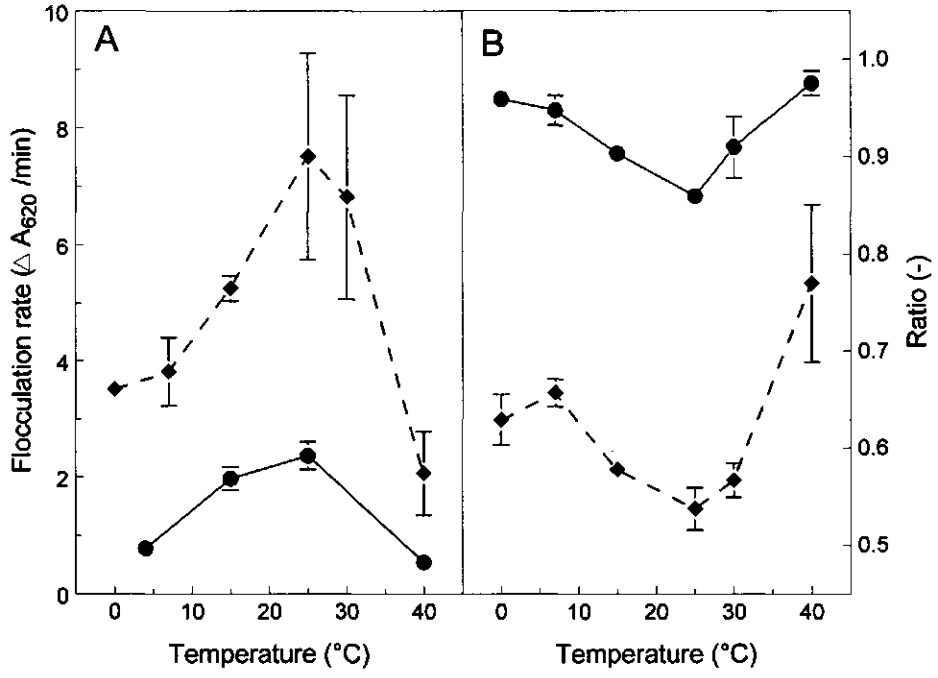

Figure 3. **Effect of temperature on flocculation. Flocculation rate was determined after incubation at different temperatures with cells grown at 25°C (●) and 4°C (◆). A. Flocculation rate (ΔA_{620}/min). B. Ratio (OD suspended cells / OD total).**

Cell surface hydrophobicity

It has been reported that an increase in cell surface hydrophobicity accompanied the onset of flocculation [7, 13, 20]. Therefore, contact angle measurements of yeast cells with water were performed. Table 1 gives the values for cells grown on two different media at different temperatures. Clearly, *Saccharomyces cerevisiae* W34 showed no increase in contact angle when cells became flocculent, indicating that cell surface hydrophobicity remained constant at the onset of flocculation. However, the increased flocculation capacity of cells grown in MYGP at 4°C compared to cells grown at 25°C coincided with larger contact angles.

Table 1. **Contact angles of flocculent and non-flocculent cells grown at high (25°C) and low (4°C) temperature in different media.**

Growth conditions Medium	Temperature (°C)	Contact angle (°) Non-Flocculent cells	Flocculent cells
MYGP	25°C	70 *	70
MYGP	4°C	94	91
Mineral medium	25°C	85	86
Mineral medium	4°C	n.d.	88

n.d. not determined .
* each data point is the mean of 10 to 15 independent measurements.

Adhesion

Adhesion was tested by measuring the decrease in OD of a cell suspension after addition of carrier material. The decrease in OD was due to attachment of cells to the particles, and not to settlement of the cells (Fig. 4A). Effect of medium composition on adhesion to the DEAE carrier is shown in Fig. 4B. Clearly, attraction between the negative cell wall and the positive carrier surface resulted in a good adhesion in water. When the experiment was performed in MYGP or wort, shielding of charged groups on the carrier, most likely by medium components, decreased adhesion significantly. Small differences were observed when adhesion was tested in fresh or in spent media.

To determine the influence of flocculation capacity on adhesion, both flocculent and non-flocculent cells were incubated with carrier particles. It appeared that in complex medium, flocculating ability enhanced the adhesion to carrier 2.5 and 4.1-fold for cells grown at 4°C and 25°C, respectively. This increase could not be explained by entrapment of flocs by carrier particles, since upon mixing of a flocculating cell suspension in fresh medium, flocs were readily dispersed and adhesion occurred by the single cells (Fig. 4A).

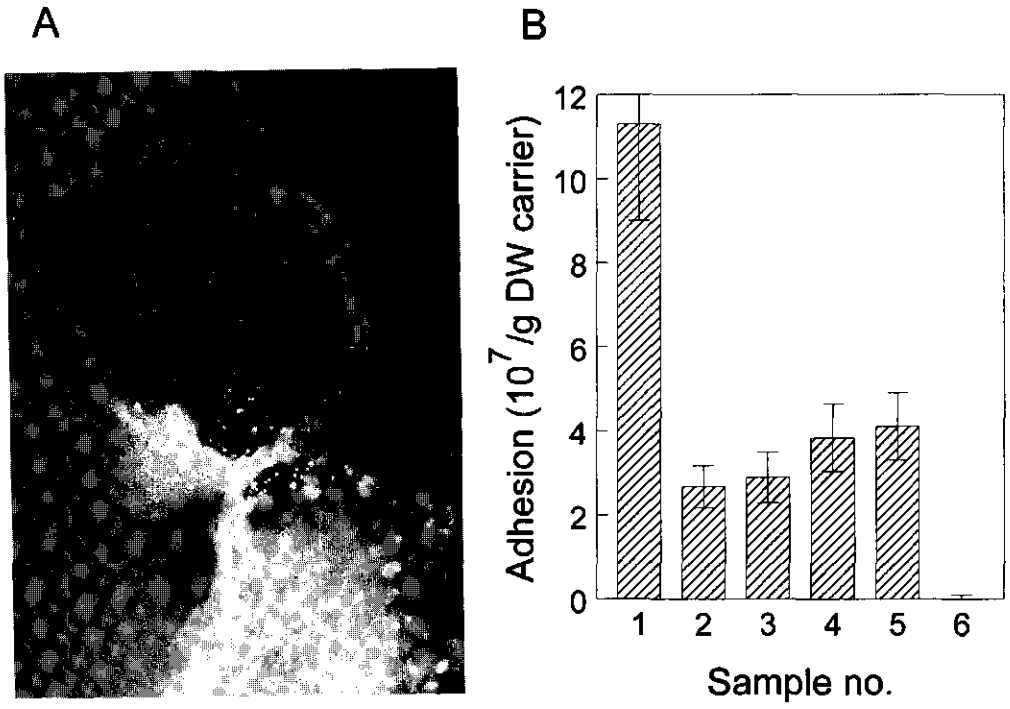

Figure 4. **Adhesion of flocculent cells to DEAE-cellulose carrier. A. Yeast cells were grown at 4°C in MYGP for 320 h., harvested, fluorescently labelled and attached to carrier particles (magnification 100-fold). B. Adhesion of cells measured by OD-decrease. Experiments were performed in; 1. Demineralized water. 2. Wort. 3. Wort + 10% (v/v) ethanol. 4. Spent media of stationary cultures in MYGP and 5. Green beer. 6. Control (no carrier).**

To determine the immobilization of cells during production of alcohol-free beer, carrier material was taken and analyzed by fluorescent staining of the yeast cells. After a production period of approximately one month, a high number of cells was found on the carrier (Fig. 5B). Due to slow steady growth at low temperature the amount of yeast cells increased to high numbers (approximately 3 10^9 cells g^{-1} carrier).

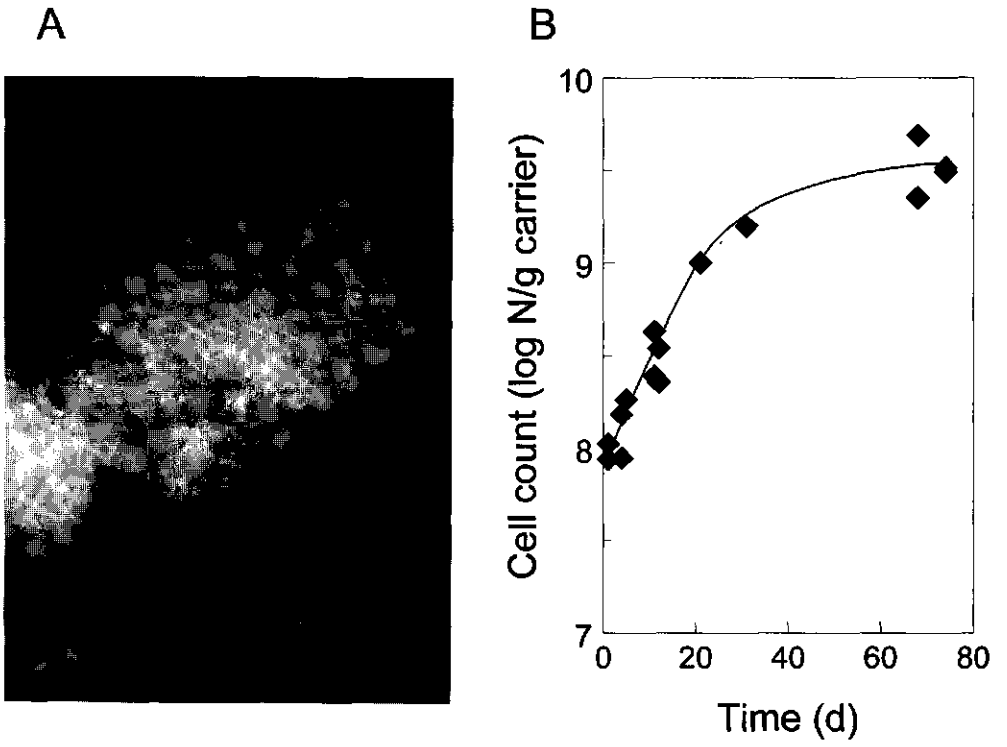

Figure 5. **Immobilization of *S. cerevisiae* W34 to DEAE-carrier during production of alcohol-free beer. A. Fluorescently labelled yeast cells immobilized to a carrier particle (magnification 100-fold). Sample was obtained four weeks after start of the alcohol-free beer production. B. Growth of *S. cerevisiae* W34 during production of alcohol-free beer.**

Discussion

Flocculation characteristics

Saccharomyces cerevisiae W34 flocculates according to the NewFlo phenotype. The main characteristics of a lectin-type flocculation are present, i.e. the flocculation is calcium dependent, mannose sensitive, and inhibited by proteases and higher temperatures. No increase in cell wall hydrophobicity was observed with cells of *S. cerevisiae* W34. On the basis of our data, and the literature available on this subject [4, 5, 7, 13, 19], we conclude that the increase in cell

wall hydrophobicity at the onset of flocculation is not a general characteristic of lager yeast strains, but is strain-dependent.

Medium composition has a strong influence on the flocculation behaviour. The exact onset of flocculation changes when cells are grown in a defined mineral medium (cf. Fig. 1 and 2), and flocculation capacity is temporary and only very weak. Flocculation observed in a defined medium is a controversial phenomenon. Stewart *et al.* [15] claimed that all lager yeast strains flocculate well in a defined, glucose-ammonium medium, whereas Teunissen and Steensma [21] and Stratford [17], observed the opposite. The latter claimed that low pH values may cause the inhibition of flocculation, as was also mentioned by Soares *et al.* [14]. In our experiments, this low pH value may also be the inhibitor of flocculation at prolonged growth. The minimal medium is not buffered, thus acidity drops to values of pH 3, whereas in complex media the decrease in pH is to approximately 4.

Effect of growth temperature

A significant difference is shown in the cell wall hydrophobicity of yeast grown at different temperatures; contact angle measurements indicate that low temperature cells have a more hydrophobic cell wall. In literature, only effects at higher temperatures were reported. Hazen *et al.* [6] compared data obtained at 37°C to 24°C: total cell wall hydrophobicity remained constant, however a difference in distribution of hydrophobicity over the cell wall was observed.

The higher cell wall hydrophobicity at low growth temperature coincides with an increase in flocculation, i.e. flocculation rate is increased, and the concentration of residual suspended cells is decreased. An increase in rate indicates formation of bigger flocs. These will sediment faster, resulting in a higher rate of OD decrease. At a constant total cell concentration, floc size is directly proportional to the inter-yeast bond strength [18]. In addition, the decrease in the concentration of single cells remaining in suspension also indicates an increase in bond strength. In part, these tighter bonds may be explained by the increase in hydrophobicity. However, other factors like formation of lectins and receptors are probably more important, as Van Hamersveld *et al.* [23] calculated that physical interactions such as hydrophobicity can not account for the bond strength between cells in a floc. Cell-cell bonds are primarily based on lectin-receptor interactions.

Effect of assay temperature

The optimum temperature for flocculation is 25°C (Fig. 3). Flocculation rate is highest, and amount of single suspended cells is lowest, indicating that the bond strength between yeast cells has its optimum at 25°C. Since lager yeast strains are generally grown and adapted to much lower temperatures, the decrease in bond strength at higher temperatures may be caused by extraction of

lectins from the yeast cell wall. The optimum temperature for flocculation is significantly higher than the 15°C optimum observed by Van Hamersveld *et al.* [24]. Apparently, temperature optimum is strain dependent and can vary significantly.

Adhesion

Adhesion of cells to a surface is a general feature of micro-organisms. However, the phenomenon is hardly exploited in modern food biotechnology. Production of alcohol-free beer [9, 25] and maturation of green beer [8] are both processes that use cell adhesion; in these cases immobilization to a DEAE-cellulose based carrier. It is thought that adhesion occurs by charge differences between DEAE-cellulose and the yeast cell wall. Whereas no significant difference was observed between the cell surface charge of flocculent and non-flocculent cells, as measured by zeta potential measurements (data not shown), we observed that flocculation capacity increased the adhesion of *S. cerevisiae*. This indicates that adhesion is more complex than mere charge interactions. In a complex medium such as wort, all kinds of components will bind to the matrix, limiting the number of charged residues for interaction with cells. A shielding effect on the surface charges can be expected, and other characteristics of the carrier may become involved in binding. Since it contains polystyrene, part of the carrier has a hydrophobic nature, which may enable interactions with hydrophobic cell wall components. In addition, the carrier has a rough surface. Pockets in the carrier will enable settlement of cells. Once settled, they may grow out to form micro-colonies as can be seen in Fig. 5A.

Conclusions

The flocculation process of yeast is significantly influenced by environmental conditions such as temperature, calcium availability and medium composition. In addition, strain characteristics are important for the flocculation. Differences in the specific details of flocculation between independent strains, such as cell wall hydrophobicity, and bond-strength, demand that conditions for the flocculation assay are optimized for each specific strain. The proposals made by Van Hamersveld *et al.* [24] can be used as a guide-line.

As was shown in this article, adhesion of cells to carrier can easily be visualized by fluorescent labelling of cells with carboxyfluorescein diacetate. Adhesion of *S. cerevisiae* W34 to DEAE-cellulose is influenced by several factors. One of these is the charge difference between yeast cell wall and the carrier. Flocculation capacity of the yeast, which increases the adhesion to DEAE-cellulose, is another significant factor.

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CONCLUDING REMARKS

We have investigated effects of low temperature and immobilization on the metabolism of *Saccharomyces cerevisiae*. Both fundamental aspects of cell metabolism as influenced by temperature and immobilization, as well as the practical implications for beer flavour formation were studied.

In this chapter, we will discuss the use of immobilization techniques in beer production. Several immobilized systems for the production of beer were proposed although, due to the complexity of beer fermentation, only few have been developed beyond the laboratory stage. Finally, novel and future developments in beer production will be discussed.

Theoretical implications of immobilization

Several immobilized systems have been introduced in beer fermentation, however, the number of production systems on industrial scale is limited [3, 23] (Chapter 1). Immobilization of viable cells for application in the food and beverage industry offers several advantages to traditional batch processes, such as enhanced fermentation productivity, reduced downstream processing costs, cell stability, increased process control [13, 14, 16]. However, since immobilization substantially influences cell physiology, it is necessary to gain more knowledge before the immobilized system will have its break-through in primary fermentation.

In order to become a commercial success, the immobilized system must have clear advantages over the traditional process. Its increased complexity has to be balanced by e.g. increased productivity, preferably without any changes in the end-product. Some requirements for the commercial feasibility of immobilized systems are summarized in Table 1.

In the traditional process, fermentation can be divided into three phases. Immediately after pitching there is a short semi-aerobic phase, in which yeast cells are supplied with oxygen and substrate to stimulate cell growth. In the second anaerobic phase, growth continues slowly, and fermentation rate starts to increase. When mono- and disaccharides have been taken up, cells start to flocculate and sediment. In the last phase, the maturation, diacetyl and 2,3-pentanedione are reduced. To simulate the various phases within one continuous process, an aerobic stage is to be integrated in the process. This may be realised in the form of a propagation vessel in which the yeast is handled prior to the main fermentation. Also, yeast growth and metabolism may be boosted by regular aerobic intervals.

Table 1. **Requirements of immobilized systems for application in beer production** [17, 25].

Requirements	Parameters
Quality	Proper flavour profile
	Low off-flavours, such as VDK
	Uptake of amino acids to sufficiently low levels
	No yeast autolysis
	Control of contamination
Productivity	Long lifetime
	High productivity. i.e. high through-put vs. low yeast reproduction
	High volumetric biomass concentration
	Complete attenuation
Operational control	Simple and continuous operation
	Short start-up procedure
	High flexibility, stand-by procedures needed
	Simple cleaning and sterilization

Cell growth, although by itself resulting in economical losses, is essential for the production of beer for several reasons. Flavour production of growing cells is significantly different from the flavour production of non-growing cells (Chapter 5). Also, with growth amino acids are removed from the wort, which otherwise in a later phase would give flavour differences. Furthermore, with cells in a healthy state, less autolysis will occur and a vivid yeast culture is, up to a certain limit, capable of out-numbering spoiling organisms. However, extended cell growth in immobilized systems increases the risk of diffusion limitations of substrate or product and the lifetime of a reactor is limited in those cases in which cells cannot be washed out of the system. Gel materials in which the cells are entrapped, should therefore be avoided. Cells should at best be bound to a surface, or contained in suspension behind some kind of barrier. To limit yeast growth, the temperature of the fermentation may be lowered. Experiments in the reactor (Chapter 6) and in batch cultures (Chapter 2) however, showed the ability of this brewer's yeast strain to maintain growth at very low temperatures. Cells are able to metabolize glucose and to reduce numerous wort components to alcohols and esters even at temperatures as low as 0 to 4°C (Chapter 4).

During the primary fermentation of beer, the conversion of sugar to ethanol is rapid in the presence of sufficient numbers of cells (Chapter 2). However, both rate and extent of attenuation need a careful control. When a fermentation is too strong flavour defects may arise. Contrary, a slow process is economically not interesting. By adjusting the temperature and the cell concentration, fermentation rate and formation of flavours can be controlled (Chapter 5). Low temperatures will on one hand have the disadvantage of a slow

fermentation, although this may be compensated by an increase in the number of yeast cells in the immobilized system. On the other hand, low temperatures will have the advantage of less diacetyl production, and thus less flavour defects (Chapter 5).

In the maturation stage, VDKs are reduced to less flavour-active components [12]. In immobilized systems, high cell numbers may be used to speed up this process. In system design, one has to take this aspect into consideration. Integration of maturation in the system is possible by lowering the temperature and increasing the residence time. However, one may also think of a separate system consisting of a packed-bed reactor in which high cell numbers mature beer in a short time period. Such a system is ideally preceded by heat-treatment to convert α-acetolactate to diacetyl [3, 11].

Practical implications of immobilization

A variety of immobilization techniques is available. These can basically be divided in 4 groups; viz. adsorption, flocculation, entrapment and containment. The choice for a specific immobilization technique and carrier, as well as the development of the reactor depend on fermentation parameters such as mass transfer requirements, increase of biomass, CO_2 evolution and productivity. So far, two reactor types have been described in literature which were used in beer fermentation. In addition several combinations of reactor systems were developed.

Packed bed reactor

Advantages of packed bed reactors are their simple design and low energy requirements [25]. In addition, a high biomass to volume ratio can be reached. However, poor liquid mixing may result in formation of liquid channels in the bed, stimulated by a build-up of gas pockets. Furthermore, yeast growth may result in a pressure increase and eventually a blockage of the flow. Therefore, packed-bed reactors are generally used only in processes with a short residence time, such as maturation of green beer or production of alcohol-free beer.

DEAE-cellulose

Industrial scale packed bed reactors are used for the production of alcohol-free beer (Chapter 1) and maturation of green beer [11]. In both cases, the carrier consists of polystyrene coated with DEAE-cellulose. In the case of maturation of green beer, the reactor is preceded by a heat-treatment unit, which accelerates the chemical carboxylation of α-acetolactate to diacetyl. The latter is then reduced by the immobilized yeast in the reactor during a residence time of 2 to 3 hours.

Primary fermentation with a packed bed reactor and DEAE-cellulose as carrier was also investigated [8]. With the reactor a 6-fold increase in productivity was reached when compared to traditional batch fermentation. Although concentrations of flavours in the end product were comparable, an average of 20% dead cells in the immobilized reactor was reported.

Porous glass

A similar maturation system has been developed with porous glass beads as carrier [3]. Advantage is the possibility of steam sterilization of the reactor. Extended growth of yeast cells in the pores of the glass will however increase the risk of cell death and autolysis, due to diffusion limitations of substrate.

Two packed-bed fermentors in series, separated by a buffer vessel, were used to perform primary fermentation [24]. Although significant variation in the outflow of the first reactor with respect to flavour formation and attenuation were observed, the green beer leaving the second reactor appeared to be little affected by this and showed much less variation.

Hollow fibers

Fibers consisting of silicon carbide have been used to form the matrix of a fixed bed as part of a loop reactor. The silicon carbide matrix has a porosity of 30%, and is characterized by an asymmetrical pore size distribution. Pores near the surface have a limited size, pores within the matrix can be up to 100 µm in diameter, which enables good colony formation. Vertical positioning of the fibers enables an easy release of CO_2 from the reactor. Wort is circulated continuously through the loop reactor, and can be withdrawn from the reactor at different attenuation levels. In this way alcohol-free beer, as well as partially fermented beer can be produced [21, 22]. By placing two such fermentors in series, main fermentation was performed with high productivity. Flavour formation could be optimized by adjusting the oxygenation [1].

Fluidized bed reactor

In a fluidized bed reactor (FBR), liquid mixing is generally high which stimulates yeast metabolism and flavour production. The reactor may consist of a tall fermentor filled with a carrier material with increased density compared to the liquid. This prevents that the up-flow in the reactor flotates and removes the particles. A continuous injection of a mixture of air, N_2 and CO_2 may increase circulation. The amount of oxygen injected in the reactor will be proportional with the amount of cell growth. When using an FBR, it may be advantageous to use yeast strains with increased flocculation capacity. In this way, biomass concentration can be increased substantially when compared to normal yeast strains, which enhances the productivity of the reactor [10].

The tower fermentor

The tower fermentor, which has been used on an industrial scale for several years, can be considered as an example of an FBR, although without use of a carrier material [7]. Cells are retained in the tower fermentor by their flocculation. In the long and tall fermentor, fresh wort is continuously added at the bottom. Here, flocs will break up due to the high sugar concentrations (Chapter 6). A continuous wort stream through the reactor, which can more or less be considered as a plug-flow, takes the cells to the top of the fermentor. In the upper part of the fermentor, sugars have been consumed, which causes the cells to flocculate. In the top, a specially developed settler separates green beer from yeast cells. By careful monitoring and controlling the fermentor a balance is reached between attenuation, cell growth and removal of cells.

Cascade FBR

A fluidized bed reactor was used by Umemoto *et al.* [20]. As a carrier, chitosan beads were used, and the reactor was operated as a repeated batch. When apparent extract reached approx. 2°Plato, usually within 18 hours, the young beer was transferred to a second reactor for maturation. The complete fermentation process took about 50 hours, and the beer was claimed to be comparable to commercially available beer.

Combined systems

Due to the complexity of beer fermentation, several combined systems were developed. In combined systems, variations in the first reactor are generally diminished in a second step. Also, the design may be such that the first reactor can be considered as a continuous inoculation system for the second reactor. Advantages of each specific part can be exploited at its best, e.g. requirements of mass transfer and cell growth are more sufficiently met with an FBR or stirred tank, whereas maturation can easily be performed with a PBR.

Cascade fermentation

Stirred tanks in cascade are currently used by a New Zealand brewery [7, 17]. In a first propagation tank, a mixture of yeast and wort is continuously added and oxygen is supplied to stimulate yeast growth. This mixture flows over in subsequent stirred tanks where yeast is kept in suspension to finish the fermentation. In a final maturation tank, the suspension is cooled down and yeast is separated by gravity. Part of the yeast is recycled to the propagator in order to have precise control of fermentation rate.

CSTR and PBR

The two stage system described by Yamauchi *et al.* [26] consists of a first stage being a semi-aerobic CSTR, in which cells are stimulated to grow. In a subsequent PBR filled with porous glass beads, fermentation is continued from approx. 8° to 1.8-2.5°Plato. Beer flavour however was reported to differ significantly from traditionally fermented beer, although amino acid uptake could be controlled precisely.

FBR and PBR

A fluidized bed reactor has the advantage of thorough mixing which increases yeast growth and flavour formation. In addition, release of CO_2 does not pose significant problems as it may do in fixed bed reactors. An FBR was investigated by Tata *et al.* [19], who compared its performance with a loop reactor containing porous silicon carbide cartridges. Both systems produced elevated levels of vicinal diketones compared to a conventional fermentation. To remove these, the young beer was heat-treated and maturated with a packed bed reactor. With slightly lower levels of fusel alcohols, and higher levels of esters, beer of acceptable flavour was produced.

Future aspects.

Wort composition and production

Despite significant effort, beer fermented with an immobilized system will taste differently when compared to traditionally fermented beer. Part of these changes may be counteracted by a change of wort composition. Special worts will have to be developed with e.g. lower concentrations of amino acids. Furthermore, the use of high gravity worts will have the advantage of increased ester formation during fermentation, which compensates for the lower amounts usually reached at low temperature.

Immobilized fermentation systems need a continuous feed of sterile wort. This can be realized by installing large buffer tanks in front of the fermentor. However, continuous production of wort is a more attractive alternative. For that purpose, further developments in e.g. cross-flow filtration of mash and wort, and continuous boiling systems will be necessary.

Assimilation

Beer fermentation can be divided into an aerobic growth phase, and an anaerobic fermentation phase. With the introduction of the assimilation unit, these phases have been more or less separated. Back *et al.* [2] developed a repeated-batch system based on the traditional propagation process. Wort is

fermented with continuous injection of oxygen/air. Before fermentation is finished, usually at approximately 50% attenuation level, the major part of the yeast is removed towards the classic beer fermentation, while the remainder is filled up with fresh wort. Higher efficiency and increased cell vitality and viability were claimed to be the result of introduction of these units into breweries.

Novel yeast strains

In addition, novel yeast strains may be used to compensate for the changes in the composition of beer from immobilized systems. These may be developed by classic genetic techniques or by genetic engineering. Some concerns of the brewing industry against genetic engineering were satisfied by a technique developed by the Carlsberg brewery [18]. No foreign genes were introduced into the yeast genome, and the plasmid vector and the antibiotic resistance genes which it contains were eliminated during deletion of *met10* in a wild-type brewer's yeast [6]. The gene product of *met10* is involved in sulfate metabolism; deletion resulted in increased sulfite concentrations during fermentation, which improved the flavour stability of the beer.

Other examples of novel yeast strains bred by genetic manipulation or classic methods are strains with decreased H_2S production and an increased ability to ferment dextrins [5, 9]. Especially for immobilized systems, yeast strains may be developed which have an improved flavour production. An increase in acetate ester synthesis is e.g. possible by introduction of multiple copies of the alcohol acetyl transferase gene (*atf1*) [4]. Furthermore, yeasts in immobilized systems generally produce higher levels of α-acetolactate. This can be prevented by introduction of the acetolactate decarboxylase gene into brewing yeasts, which readily converts this precursor to diacetyl. An alternative approach is to alter enzyme levels in the metabolic pathway to valine. Increasing levels of the *ilv5* gene product, which converts α-acetolactate to dihydroxy isovalerate, showed significant decrease in diacetyl levels due to a higher flux of α-acetolactate to valine [15].

Productivity in immobilized systems is directly proportional with the number of yeast cells present. It therefore will be interesting to use yeast strains with an enhanced flocculation capacity. A further productivity increase is possible with an enhanced flux of glucose through glycolysis. An improved reduction capacity may be interesting in case of alcohol-free beer production. Increasing the activity of a reducing enzyme such as the bcADH would have no effect on ethanol production since activity of this enzyme with acetaldehyde is low (Chapter 3).

In conclusion

The examples mentioned above show that despite a history of over 5000 years new developments are still being introduced in beer production. In the future, combinations of new processes and novel yeast strains will increase the versatility and quality of the product beer. Whether or not these new developments will be introduced will depend on both economic factors, and marketing arguments such as consumer's approval of the use of novel yeast strains. The current increase in the consumer's demand for specialty beers will certainly have a beneficial effect on new developments in beer brewing.

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SUMMARY

A system for production of alcohol-free beer is described. A limited fermentation is carried out with immobilized cells of a brewer's yeast strain of *Saccharomyces cerevisiae* var. *uvarum* in a packed bed reactor. Combined stress factors such as low temperature (0-4°C) and anaerobic conditions limit cell metabolism. Of the available sugars only a small amount of glucose is metabolized, resulting in low concentrations of ethanol (<0.1%). Flavour compounds are formed by reduction of wort aldehydes, as well as reduction of intracellular metabolites.

The objective of the present study was to gather information on the physiology of the yeast under the conditions used for alcohol-free beer production. It appeared that growth occurred at temperatures of -2°C to 30°C, with long lag times at low temperatures (Chapter 2). This indicated that production periods with the packed bed reactor are limited, since growth of the yeast will eventually block the flow. Subsequently, sugar metabolism was analyzed in cells pregrown at 25°C or at 4°C. Surprisingly, similar rates of sugar metabolization were found, although high or low temperature grown cells were subjected to an upward or downward temperature shift to the assay temperature of 4 or 25°C. In addition, energy levels analyzed by ATP measurements were high in both types of cells. The observed increase in lag time at very low growth temperatures may thus be caused by the cold-shock response which is known to cause an arrest in overall protein synthesis, although it stimulates synthesis of cold-shock proteins.

Metabolism was further monitored by respiration measurements and product formation using cells grown at high or low temperature. In cells grown at 25°C, but not in cells grown at 4°C, substrate-induced respiration was observed upon addition of fermentable sugar or ethanol. By fluorescent labelling with the mitochondrial dye DASPMI, intact mitochondria were observed in both types of cells. In addition, an increase in glycerol formation was observed which may be caused by the role of glycerol formation in the redox balance of the yeast cells. With cells grown at 4°C biomass yield was significantly decreased; both final levels of cfu's reached at stationary growth and the biomass yield per mole glucose during exponential growth were reduced. Furthermore, *in vitro* hexokinase activity was increased and, since hexokinase has been shown to be involved in glucose repression, this may be linked to a higher degree of glucose repression in 4°C grown cells.

In alcoholic fermentations, activity of the ADHs is not limited to acetaldehyde. Several so-called Strecker aldehydes, formed by transamination and subsequent decarboxylation of amino acids, are reduced to fusel alcohols.

During production of non-alcohol beer, an optimum has to be found in the reduction of wort aldehydes, which themselves cause off-flavours, and limited production of ethanol and flavours by fermentation. It had appeared that ADH1 is not involved in reduction of higher aldehydes since no correlation was found between reduction of these compounds and the activity of this enzyme in extracts of cells obtained during different stages of the alcohol-free beer production. The involvement of additional ADHs in reduction of branched-chain aldehydes, such as 2- and 3-methylbutanal, was therefore investigated. In preliminary experiments we observed that, compared to an aerobic environment, anaerobic conditions resulted in a significant increase in NADP-dependent *in vitro* reduction. In Chapter 3, we report the purification and characterization of a novel NADP-dependent branched-chain alcohol dehydrogenase (bcADH) from the brewer's yeast *S. cerevisiae*, expressed under anaerobic conditions. The enzyme prefers branched-chain aldehydes such as 3-methylbutanal as substrate, and has an increasing catalytic efficiency for substrates with increasing carbon chain length. It prefers NADPH as the electron donor, but at low ionic strength (I = 0.01 M) it is capable of using NADH, although affinity is substantially lower than that for NADPH. Since *S. cerevisiae* lacks transhydrogenase-activity, which catalyses the conversion of NADH into NADPH or vice versa, the bcADH may play a role in balancing the redox value for NADPH/$NADP^+$ under anaerobic conditions. Based on the high reductive activity with 3-methylbutanal, especially at physiological pH and ionic strength of 0.1 M, we suggest that the enzyme has an important function in reduction of Strecker aldehydes and thus may positively affect flavour composition during alcoholic fermentation.

In Chapter 4, the activity of the bcADH was monitored during alcohol-free beer production. Furthermore, we investigated the influence of immobilization of yeast to DEAE-cellulose on sugar fermentation and aldehyde reduction. Immobilized cells showed higher activities of hexokinase and pyruvate decarboxylase compared to cells grown in batch culture. In addition, a higher glucose flux was observed, with enhanced excretion of main fermentation products, indicating a reduction in the flux of sugar used for biomass production. ADH activity was higher in immobilized cells compared to that in suspended cells. However, during production a decrease was observed in NAD-specific activity. This could be explained by glucose repression of ADH2, which is present at high levels in stationary cells but is repressed when cells are growing in the presence of glucose. Opposite to ADH2, NADP-specific ADH activity increased in the immobilized cells during alcohol-free beer production. The shifts in enzyme activities and glucose flux correlate with a higher *in vivo* reduction capacity of the immobilized cells. We thus conclude that immobilization itself improves reduction during the production of alcohol-free beer.

Likewise, ester formation and physiology of immobilized yeast cells in the bioreactor were investigated. During production, a simultaneous increase in the activity of alcohol acetyl transferase, the main acetate ester synthesising enzyme, and formation of ethyl acetate and isoamyl acetate were observed (Chapter 5). In addition, the relative amount of unsaturated fatty acids decreased significantly, whereas squalene, which is an intermediate in the synthesis of sterols prior to the reaction step with molecular oxygen, accumulates. Since these phenomena coincided with a significant decrease in growth rate, we conclude that the anaerobic conditions, and the absence of substantial levels of unsaturated fatty acids in wort, limit cell growth during production. The decrease in unsaturated fatty acids will subsequently stimulate formation of acetate esters.

Generally, no diacetyl formation is observed during alcohol-free beer production, although activity of acetohydroxy acid synthase (AHAS) was constant at the entire production period. It appeared that the low temperatures (2°C) suppressed production of diacetyl-precursor, whereas at 12°C substantial levels were obtained.

The observations mentioned above enable the brewer to optimise the flavour profile of the final product. A balance must be reached between flavour formation and reduction on one hand, and growth and ethanol formation on the other. Anaerobic conditions inhibit growth and stimulate ester production, but yeast cells need oxygen for synthesis of essential cell components. Oxygen however also stimulates growth and may cause oxidative off-flavours. By introduction of regular intervals of aeration, an optimum can be reached between the supply of oxygen for yeast metabolism, and the prevention of oxidation of the alcohol-free beer. By varying the length of the interval, and by variation of temperature and flow rate, concentrations of ester compounds in the end-product can be controlled during the production period.

The immobilization of *S. cerevisiae* to DEAE-cellulose is a gentle process. In Chapter 6, we investigated whether flocculation characteristics of the yeast cells had an influence on its immobilization. When grown in wort, cells developed flocculation at the end of exponential growth, according to the NewFlo phenotype. In defined medium, the flocculation capacity appeared to be temporary, and was rapidly lost during the stationary phase. This was explained by the low pH of the medium which is reported to decrease flocculation capacity in *S. cerevisiae* strains. In addition, flocculation was further characterized by measuring cell wall hydrophobicity. No increase in hydrophobicity was observed at the onset of flocculation, however a correlation between high flocculation capacity and high cell wall hydrophobicity existed in cells grown at different temperatures. Low growth temperatures (4°C) increased flocculation capacity approximately four-fold, compared to growth at high temperatures (25°C), although, with both types of cells, the optimum temperature for flocculation was 25°C.

Adhesion of cells to DEAE-cellulose carrier was analyzed with a novel method using carboxyfluorescein-stained cells. This method also allowed rapid analysis of immobilization during the alcohol-free beer production. Adhesion of *S. cerevisiae* W34 to DEAE-cellulose is influenced by several factors. One of these factors is the charge difference between the yeast cell wall and the carrier, and the concentration of ions in solution. Since a high flocculation capacity stimulated adhesion to the DEAE-cellulose carrier, this capacity appeared to be another important factor.

For several years now, the system described in this paper has been used for the production of alcohol-free beer. Further applications are the secondary fermentation of green beer, and biological acidification of wort with lactic acid bacteria. Whether novel immobilized systems will be introduced in primary beer fermentation depends on several factors. Fermentation is a complex process and if its different phases are translated to several stages within one system, overall complexity will increase. However, the increased productivity of immobilized systems and their flexibility with respect to logistic issues, will prove to be superior on the long term.

SAMENVATTING

Het hier beschreven promotieonderzoek gaat over de productie van alcoholvrij bier. De doelstelling van deze studie was het vergaren van informatie over het metabolisme van de gistcellen tijdens de productie van alcoholvrij bier.

Alcoholvrij bier kan worden geproduceerd in een gepakt-bed reactor. Het bed bestaat hierbij uit een dragermateriaal van DEAE-cellulose waarop de gistcellen, *Saccharomyces cerevisiae* var. *uvarum*, zijn geïmmobiliseerd. Door de gistcellen aan verschillende stress factoren zoals lage temperaturen en anaërobe omstandigheden bloot te stellen, wordt de stofwisseling van de gistcel geremd. Hierdoor zal slechts een gedeelte van de beschikbare suikers worden omgezet, zodat een lage concentratie aan ethanol wordt verkregen (<0.1%).

In het onderzoek is de gist bij verschillende temperaturen gekweekt (Hoofdstuk 2). Bij de lage temperaturen werd nog steeds groei waargenomen; welke stopte bij temperaturen kleiner dan -2°C. Tegelijk neemt de lag-fase van de gist snel toe bij lage temperaturen.

Hoewel er een lange lag-fase werd geconstateerd in de groei, bleek dat het metabolisme in de cel niet stillag. Om de invloed van de temperatuur op de gistcellen te bepalen zijn de cellen gegroeid bij een hoge temperatuur (25°C) en een lage temperatuur (4°C). Vervolgens is gekeken naar het suikermetabolisme en de ademhaling van de cellen. Suikers werden direct opgenomen en omgezet door de cellen. Vergelijkbaar gedrag werd gevonden wanneer de cellen een temperatuurshock ondergingen van lage naar hoge temperatuur, of omgekeerd. Omzetting van de opgenomen suikers zorgde tevens voor een snelle toename van intracellulair ATP.

De ademhaling van cellen werd eveneens bij hoge (25°C) en lage (4°C) temperatuur getest. Suikers en ethanol veroorzaakten een duidelijke toename van de ademhaling bij cellen die gegroeid waren bij 25°C. Daarentegen wordt deze substraat-geïnduceerde ademhaling niet waargenomen bij cellen die zijn opgegroeid bij 4°C. Door fluorescente labeling van cellen met een kleurstof die zich specifiek in mitochondria ophoopt, blijkt dat er wel degelijk actieve mitochondria aanwezig zijn. De verminderde ademhaling duidt op een wijziging in de redoxbalans van de cellen. Een verdere aanwijzing hiervoor werd gevonden in de toename van glycerolproductie in cellen die bij 4°C waren gegroeid.

Tevens werd gevonden dat bij groei bij lage temperaturen de productie van biomassa afnam; we vonden zowel een vermindering van het aantal kve's, als een vermindering van de biomassa opbrengst per mol glucose tijdens exponentiële groei. Uitgaande van theoretische opbrengsten kan berekend worden dat de benodigde hoeveelheid groei-energie bij hogere temperaturen gedeeltelijk

uit de ademhaling moet komen, ca. 5%. Uit de berekeningen blijkt ook dat bij lage temperaturen er genoeg energie uit de glycolyse gehaald kan worden. Daarnaast is in cellen die gegroeid zijn bij 4°C een sterk verhoogde activiteit van hexokinase waargenomen. Dit wijst op een veranderde glucose-repressie in deze cellen aangezien dit enzym een belangrijke rol speelt in deze repressie. Dit kan een gedeeltelijke verklaring geven voor de gevonden fenomenen.

Tijdens alcoholische gisting is de activiteit van alcoholdehydrogenase (ADH) niet beperkt tot aceetaldehyde alleen; verschillende Strecker-aldehyden worden eveneens gereduceerd. Omdat er geen relatie werd gevonden tussen de activiteit van het primaire ADH en de reductie van deze Strecker aldehyden zijn we op zoek gegaan naar additionele ADHs. Daarbij werd één extra ADH gevonden, gezuiverd en partieel gekarakteriseerd (Hoofdstuk 3). Expressie van het enzym vindt plaats onder anaërobe omstandigheden. Het enzym prefereert vertakte aldehyden, zoals 3-methylbutanal, en de katalytische efficiëntie neemt toe naarmate de lengte van de koolstofketen langer is. NADPH wordt als electrondonor geprefereerd boven NADH. *S. cerevisiae* bezit geen enzym dat de conversie van NADH naar NADPH katalyseert (een transhydrogenase). Het bcADH kan dus eventueel een rol kan spelen in de redoxbalans van $NADPH/NADP^+$ onder anaërobe omstandigheden. Gezien de eigenschappen van het bcADH wordt verwacht dat het enzym een belangrijke rol speelt in de reductie van Strecker-aldehyden tijdens alcoholische vergistingen.

In Hoofdstuk 4 is de activiteit van het bcADH gedurende de productie van alcoholvrij bier beschreven. Daarnaast is de invloed van immobiliseren van gist op de fermentatie en reductie van aldehyden onderzocht. Geïmmobiliseerde cellen vertonen 1) hogere activiteiten van hexokinase, 2) sterkere aldehydereductie, en 3) een verhoging van de glucoseflux en de belangrijkste fermentatieproducten. Tijdens de alcohol-vrij bier productie wordt gedurende de eerste dagen een sterke afname gevonden in de NAD-specifieke ADH activiteit. Dit kan verklaard worden uit glucose-repressie van ADH2 dat in hoge concentratie aanwezig is in de stationaire cellen waarmee de reactor geënt wordt. De NADP-specifieke ADH activiteit neemt gedurende de productieperiode toe. De verschuivingen in de enzymactiviteiten en in de glucoseflux correleren met een hogere *in vivo* reductiecapaciteit van de geïmmobiliseerde cellen. Immobilisatie van de gistcellen heeft dus een positieve uitwerking op de reductie.

Naast de reductie van suikers is er tevens gekeken naar vorming van aroma-componenten, zoals esters en diacetyl. Gedurende de productie werd verhoging gevonden in de alcoholacetyltransferase activiteit, en een toename in de vorming van de esters ethylacetaat en isoamylacetaat (Hoofdstuk 5). De verschuivingen in vetzuursamenstelling duiden op een tekort aan moleculaire zuurstof; het relatieve percentage onverzadigde vetzuren nam af, en squaleen, een precursor in de synthese van sterolen, accumuleerde. De anaërobe

omstandigheden in de reactor en de afwezigheid van onverzadigde vetzuren in wort, veroorzaken daardoor een afname in groeisnelheid. De afname in onverzadigde vetzuren stimuleert de vorming van acetaatesters.

Tijdens de productie vindt er geen vorming van diacetyl plaats, ondanks de hoge activiteit van het enzym acetohydroxyzuur synthetase die wordt waargenomen. De lage productietemperaturen (2°C) onderdrukken de vorming van de diacetyl precursor α-acetolactaat. Bij de experimenten die op 12°C werden uitgevoerd, bleek dat er substantiële hoeveelheden precursor worden gevormd.

Door variatie van de intervallen van beluchtingsperioden in de reactor, de temperatuur en de flow-snelheid kan de brouwer de samenstelling van de aroma componenten tijdens alcohol-vrij bier productie optimaliseren. Temperatuur dient hierbij laag te blijven om vorming van α-acetolactaat te voorkomen.

In Hoofdstuk 6 is de invloed van het vlokkingskarakter van de gist op de immobilisatie aan DEAE-cellulose beschreven. Cellen van *S. cerevisiae* ontwikkelen vlokking aan het einde van de exponentiële groei, wanneer suiker in de oplossing is uitgeput. Bij een zeer lage pH, zoals bijv. in ongebufferd mineraal medium, verliezen de cellen de vlokkingseigenschappen weer. Er werd geen toename gevonden in de hydrofobiciteit van de celwand op het moment dat de vlokking zich optrad. Echter, cellen die bij lage temperatuur gegroeid waren, hadden een hogere celwand hydrofobiciteit, en een sterkere vlokking in vergelijking met cellen die bij 25°C waren gegroeid.

De immobilisatie van cellen aan DEAE-cellulose werd getest met een nieuwe methode die gebruik maakt van fluorescentie door carboxyfluoresceïne. Hierdoor was het tevens mogelijk de immobilisatie tijdens de productie van alcoholvrij bier te onderzoeken. De immobilisatie wordt beïnvloedt door verschillende factoren. Één van deze factoren is het ladingsverschil tussen het dragermateriaal en de celwand van de gisten. Een andere factor is de vlokking van de gist; in cellen met een hoge vlokkingscapaciteit blijkt namelijk een significante toename van de hechting aan de drager.

De gepakt-bed reactor wordt reeds gedurende een aantal jaren gebruikt voor de productie van alcoholvrij bier, rijping van jongbier, en voor de biologische zuring van wort met melkzuurbacteriën. De introductie van nieuwe geïmmobiliseerde systemen in de vergisting van bier hangt af van verschillende factoren (Hoofdstuk 7). Vergisting is een complex proces dat meerdere fasen doorloopt. Door de verschillende fasen in een continu proces te laten plaatsvinden neemt de complexiteit van het systeem toe. Door een beter inzicht in het vergistingsproces, en innovatieve ontwikkelingen zal het continu-proces verder ontwikkeld worden. De duidelijke voordelen van geïmmobiliseerde systemen, zoals de hoge productiecapaciteit en de flexibiliteit met betrekking tot de logistiek van de

productie, zullen naar verwachting van doorslaggevend belang zijn voor de uiteindelijke introductie van deze systemen.

ZUSAMMENFASSUNG

Die Herstellung von alkoholfreiem Bier kann mit einem Festbettreaktor geschehen. Das Bett besteht aus einem Trägermaterial (Carrier) von DEAE-Zellulose, auf welchem sich die immobilen Hefezellen, *Saccharomyces cerevisiae* var. *uvarum*, befinden.

Durch verschiedene Streßfaktoren wie zum Beispiel niedrige Temperaturen und anaerobe Umstände wird der Stoffwechsel der Hefezellen gebremst. Ein Teil der zur Verfügung stehenden Zucker wird umgesetzt, sodaß man eine niedrige Konzentration von Äthanol erhält. (<0,1%).

Eine der Zielsetzungen dieser wissenschaftlichen Untersuchung war das Versammeln von Informationen über die Physiologie der Hefezellen während der Herstellung von alkoholfreiem Bier. Hierzu wurde die Hefe bei verschiedenen Temperaturen gezüchtet. Auch bei sehr niedrigen Produktionstemperaturen wurde noch Wachstum wahrgenommen, die minimale Wachstumstemperatur betrug -2°C. Bei niedrigen Temperaturen nimmt die Adaptionsphase (lag-Phase) der Hefezellen schnell zu (Kapitel 2). Obwohl beim Wachstum eine lange lag-Phase festzustellen war, bedeutete das nicht, daß der Stoffwechsel der Zelle ruhte. Bei Zellen, die bei einer Temperatur von 25°C gewachsen waren und bei 4°C getestet wurden, zeigte sich daß die Zucker direkt aufgenommen und umgesetzt wurden. Vergleichbares Verhalten zeigte sich auch wenn die Zellen von einer niedrigeren in eine höhere Temperatur gebracht wurden. Die Umsetzung der aufgenommenen Zucker sorgte für eine schnelle Zunahme von intrazellulärem ATP.

Die Atmung der Zellen wurde ebenfalls bei hoher Temperatur (25°C) und niedriger Temperatur (4°C) getestet. Substrate wie Zucker und Äthanol verursachten eine deutliche Zunahme der Atmung bei Zellen, die bei 25°C gewachsen waren. Bei Zellen, welche bei 4°C gewachsen waren, wurde diese substratabhängige Atmung jedoch nicht wahrgenommen. Durch Färbung der Zellen mit DASPMI, einem fluorreszenten Farbstoff, spezifisch für Mitochondria aufhäuft, wurde bewiesen, daß tatsächlich aktive Mitochondria vorhanden waren. Die geringere Atmung spricht für eine Veränderung der Redoxbalanz der Zellen. Eine weitere Bestätigung hierfür sah man in der Zunahme der Glycerolproduktion in Zellen, welche bei niedriger Temperatur gezüchtet wurden. Außerdem wurde entdeckt, daß bei Wachstum bei niedrigen Temperaturen die Produktion der Biomasse abnahm; wir fanden während der exponentiellen Wachstumsphase eine Verringerung von sowohl der Anzahl an Zellen als auch des Biomasse-Ertrages per Mol Glukose. Ausgehend von theoretischen Erträgen konnte berechnet werden, daß die notwendige Menge Wachstumsenergie bei höheren Temperaturen teilweise aus der Atmung kommen mußte, ca 5%. Aufgrund von

Berechnungen wird davon ausgegangen, daß bei niedrigen Temperaturen diese Atmung nicht notwendig ist, weil schon aus der Glycolyse genug Energie geholt werden kann. In Zellen, die bei niedriger Temperatur gewachsen sind, nahm die Aktivität von Hexokinase stark zu. Durch die Rolle, welche dieses Enzym bei der Glucoserepression spielt, kann man möglicherweise von einer veränderten Repression in Zellen, gezüchtet bei 4°C, sprechen. Das könnte eine teilweise Erklärung für die entdeckten Phänomene sein.

In alkoholischen Gärungen ist die Aktivität von Alkoholdehydrogenase nicht allein auf Acetaldehyd beschränkt; verschiedene Strecker-Aldehyde werden ebenfalls reduziert. Weil man zwischen der Aktivität des primären ADH und der Reduktion dieser Strecker-Aldehyde keinen Zusammenhang hatte finden können, ging man auf die Suche nach additionellen ADH's.

Es wurde ein extra ADH entdeckt, gesäubert und teilweise charakterisiert (Kapitel 3). Expression des Enzyms geschieht unter anaeroben Bedingungen. Das Enzym bevorzugt verzweigte Aldehyde wie z.B. 3-Methylbutanal, die katalytische Effektivität nimmt bei einer Zunahme der Kohlenstoffkette ebenfalls zu. NADPH wird als Elektronendonor bevorzugt vor NADH, obwohl letztgenannter Stoff bei sehr niedriger Ionenstärke der Pufferlösung (I = 0.01 M) und sehr niedriger katalytischer Effektivität noch angewendet werden kann. Weil *S. cerevisiae* keine Transhydrogenase-Aktivität aufweist, ein Enzym welches die Konversion (Umkehrung) von NADH in NADPH katalysiert, kann das gereinigte bcADH eventuell unter anäeroben Bedingungen in der Redoxbalanz von NADPH/NADP eine Rolle spielen. Angesichts der Eigenschaften des bcADH erwarten wir daß das Enzym während alkoholischer Gärungen in der Reduktion der Strecker-Aldehyde einen wichtigen Beitrag liefert.

In Kapitel 4 wird die Aktivität von bcADH während der Herstellung von alkoholfreiem Bier beschrieben. Außerdem wurde auch der Einfluß der Hefeimmobilisation auf die Fermentation und Reduktion von Aldehyden untersucht.

Immobilisierte Zellen zeigen 1) höhere Aktivität der Hexokinase, 2) stärkere Aldehydreduktion, und 3) eine Erhöhung des Glucoseflusses und wichtigster Fermentationsprodukte. Während der ersten Tage der Produktion von alkoholfreiem Bier wurde eine starke Abnahme in der NAD-spezifischem ADHAktivität festgestellt. Das kann mit der Glukose Repression von ADH2 erklärt werden, welches in hohem Maße in den stationären Zellen vorhanden ist, mit welchen der Reaktor geimpft wurde.

Die NADP-spezifische ADH-Aktivität nahm während der Produktionsperiode zu. Die Verschiebungen in den Enzymaktivitäten und dem Glucosefluß korrelieren mit einer höheren *in vivo* Reduktionskapazität der immobilisierten Zellen. Immobilisation der Hefezellen hat also eine positive Auswirkung auf die Reduktion.

Nicht nur nach der Reduktion wurde geschaut, auch die Bildung von anderen Aromastoffen, wie z.B. Ester und Diacetyl wurde untersucht. Während der Produktion wurde eine Zunahme in sowohl der Aktivität der Alkohol-Acetyl-Transferase festgestellt als auch die Bildung der Ester Äthylacetat und Isoamyl Acetat (Kapitel 5).

Verschiebungen in der Fettsäurezusammenstellung zeigten, daß ein Defizit an molekularem Sauerstoff auftrat; der relative Prozentsatz ungesättigter Fettsäuren nahm ab und Squalene, ein Precursor in der Synthese von Sterolen, akkumulierte. Die anaeroben Bedingungen im Reaktor und das Fehlen von ungesättigten Fetsäuren in der Würze verursachen also eine Abnahme der Wachstumsgeschwindigkeit. Die Abnahme von ungesättigten Fettsäuren stimmuliert die Entstehung von Acetat Estern.

Während der Produktion gibt es keine Bildung von Diacetyl, obwohl eine hohe Aktivität des Enzymes Azetohydroxysäure Synthetase festgestellt wurde. Die niedrigen Produktionstemperaturen (2°C) unterdrückten die Bildung des Diacetyl Precursors α-Acetolactat. Wenn die Experimente bei 12°C ausgeführt wurden, formten sich nämlich substantielle Mengen α-Acetolactat.

Durch Variation der Belüftungsintervalle im Reaktor, der Temperatur und der Durchflußgeschwindigkeit kann der Brauer die Zusammenstellung der Aromacomponenten während der Produktion von alkoholfreiem Bier optimalisieren. Die Temperatur muß jedoch niedrig bleiben, um die Entstehung von α-Acetolactat zu verhindern.

Immobilisation von *S. cerevisiae* an DEAE-Zellulose ist ein milder Prozeß. In Kapitel 6 beschreiben wir den Einfluß des Flockungskarakters der Hefe auf diese Immobilisation. Zellen von *S. cerevisiae* flockulieren am Ende ihres exponentiellen Wachstums, wenn die Zucker in der Lösung erschöpft sind. Bei einem sehr niedrigen pH wie zum Beispiel ungepufferten Mineral Medium, verlieren die Zellen diese Eigenschaften wieder. Zum Zeitpunkt daß sich die Flockung entwickelte, wurde keine Zunahme in der Hydrophobizität der Zellwand festgestellt. Jedoch, Zellen die bei einer niedrigen Temperatur gewachsen waren, wiesen eine höhere Zellwand-Hydrophobizität auf als Zellen, welche bei einer Temperatur von 25°C gezüchtet wurden. In allen Fällen zeigten die Zellen bei einer Testtemperatur von 25°C die stärkste Flockung.

Immobilisation von Zellen an DEAE-Zellulose wurde mit einer neuen Methode getestet, welche die Fluoreszenz mit Carboxyfluorescin benützt. Hiermit war es außerdem möglich, die Immobilisation auch während der Produktion von alkoholfreiem Bier zu untersuchen. Es stellte sich heraus, daß die Immobilisation von verschiedenen Faktoren beeinflußt wurde. Einer dieser Faktoren ist der Ladungsunterschied zwischen dem Trägermaterial und der Zellwand der Hefen. Ein anderer Faktor ist die Flockung der Hefe; in Zellen mit einer hohen Flockungs-

kapazität konstatierte man eine signifikante Zunahme der Haftungsintensivität an den Träger.

Während einer Anzahl von Jahren wurde der Festbett-Reaktor für die Produktion von alkoholfreiem Bier, die Reifung von Jungbier und für die biologische Säuerung der Würze mit Milchsäurebakterien benützt. Ob neue immobilsierte Systeme in der Biergärung eingeführt werden, hängt von mehreren Faktoren ab (Kapitel 7). Vergärung ist ein Prozeß, der mehrere Phasen durchläuft. Wenn man diese Phasen als einen kontinuellen Prozeß übersetzt, dann wird die Komplexität der Systeme zunehmen. Durch innovative Entwicklungen muß diese Komplexität neutralisiert werden. Die hohe Produktivität von immobilisierten Systemen und die Flexibilität in Bezug auf die Logistik der Produktion, wird auf die Dauer, so erwartet man, deren Überlegenheit beweisen.

CURRICULUM VITAE

Momenteel is ondergetekende werkzaam bij Bavaria als project manager R&D voor mouterij en brouwerij. Voorafgaand aan deze baan, die is aangevangen op 17 februari 1997, werkte hij aan onderhavig proefschrift op de toenmalige vakgroep Levensmiddelentechnologie, sectie levensmiddelenchemie en -microbiologie aan de Landbouwuniversiteit te Wageningen. Het onderzoek werd uitgevoerd in de periode 1 februari 1992 - 5 september 1996.

Zijn studie, Moleculaire Wetenschappen begonnen aan de Landbouwhogeschool in 1985, werd eind 1991 succesvol afgesloten. Hij had daarbij 2 afstudeervakken gevolgd bij respectievelijk prof. C. Heyting van de vakgroep Erfelijkheidsleer, en bij dr T. Visser van de vakgroep Biochemie. Tussendoor werd een stage uitgevoerd op het Medisch Biologisch Laboratorium - TNO te Rijswijk bij dr A. van der Drift.

In 1985 heeft hij zijn eindexamen succesvol afgelegd op het Mgr. Zwijsen College te Veghel, en daarbij zijn diploma Atheneum-ß behaald. 18½ Jaar daarvoor was hij, Martijn van Iersel, geboren op 20 oktober 1966 te Sint-Oedenrode.

LIST OF PUBLICATIONS

1. **Van Iersel, M.F.M., F.M. Rombouts and T. Abee.** Growth and physiology of *Saccharomyces cerevisiae* at low temperatures. Submitted for publication.
2. **Van Iersel, M.F.M., E. Brouwer-Post, F.M. Rombouts and T. Abee.** Influence of immobilization on fermentation and aldehyde reduction during the production of alcohol-free beer. Accepted for publication in Enz. Microb. Technol.
3. **Van Iersel, M.F.M., B. van Dieren, F.M. Rombouts and T. Abee.** 1999. Flavour formation and cell physiology during the production of alcohol-free beer with immobilized *Saccharomyces cerevisiae*. Enz. Microb. Technol. **24**: 407-411.
4. **Van Laarhoven, H., M. van Iersel, F. Dekkers, and A. Douma.** 1999. A convenient test for the determination of barley batch germination characteristics: Case studies in the malthouse. Proc. Eur. Brew. Conv. **27**: *in press.*
5. **Van Iersel, M.F.M, E. Meersman, M. Arntz, F.M. Rombouts, and T. Abee.** 1998. Effect of environmental conditions on flocculation and immobilization of brewer's yeast during production of alcohol-free beer. J. Inst. Brewing **104**: 131-136.
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7. **Favier, J.P., D. Bicanic, P. Helander, and M. van Iersel.** 1997. The optothermal approach to a real time monitoring of glucose content during fermentation by brewer's yeast. J. Biochem. Biophys. Methods. **34**: 205-211.
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